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DOCKET NO.: ISIS-4502

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:
Richard H. Tullis

Confirmation No.: 9155

Application No.: 08/078,768

Group Art Unit: 1631

Filing Date: June 16, 1993

Examiner: James Martinell

For: Oligonucleotide Therapeutic Agent And Methods Of Making Same

EXPRESS MAIL LABEL NO: EL 998517913 US
DATE OF DEPOSIT: October 18, 2004

EL998517913US

MS Appeal Brief - Patent
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF TRANSMITTAL PURSUANT TO 37 CFR § 41.37

Transmitted herewith is the APPELLANT'S SUBSTITUTE APPEAL BRIEF in this application with respect to the Official Communication received from The United States Patent and Trademark Office on **September 15, 2004**.

☒ Also transmitted herewith:

- ☒ Response to Official Communication Dated September 15, 2004
- ☒ Copy of Petition Under 37 CFR §1.181
- ☒ Appellant's Substitute Appeal Brief Pursuant to 37 CFR §41.37.
- ☒ Exhibits 1-23 cited in Substitute Appeal Brief.

☐ The fee for the Appeal Brief was paid upon filing of the initial Appeal Brief, therefore no fee is due for the Appeal Brief at this time. However, if this is deemed to be inaccurate, please charge any deficiency or credit any overpayment to Deposit Account No. 23-3050.

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DOCKET NO.: ISIS-4502 **PATENT**

☒ Petition is hereby made under 37 CFR § 1.136(a) (fees: 37 CFR § 1.17(a)(1)-(4) to extend the time for response to the Office Action of September 15, 2004 to and through November 15, 2004 comprising an extension of the shortened statutory period of One (1) month(s).

	SMALL ENTITY		NOT SMALL ENTITY	
	RATE	FEE	RATE	FEE
☒ APPEAL BRIEF FEE	$170	$	$340	$340.00
☒ ONE MONTH EXTENSION OF TIME	$55	$	$110	$110.00
☐ TWO MONTH EXTENSION OF TIME	$215	$	$430	$
☐ THREE MONTH EXTENSION OF TIME	$490	$	$980	$
☐ FOUR MONTH EXTENSION OF TIME	$765	$	$1530	$
☐ FIVE MONTH EXTENSION OF TIME	$1040	$	$2080	$
☒ LESS ANY FEE ALREADY PAID	minus	($)	minus	($165.00)
TOTAL FEE DUE		$0		$285.00

☐ A check in the amount of **<u>$.00</u>** is attached. Please charge any deficiency or credit any overpayment to Deposit Account No. 23-3050.

☒ Please charge Deposit Account No. 23-3050 in the amount of **<u>$285.00</u>**. This sheet is attached in duplicate.

☒ The Commissioner is hereby requested to grant an extension of time for the appropriate length of time, should one be necessary, in connection with this filing or any future filing submitted to the U.S. Patent and Trademark Office in the above-identified application during the pendency of this application. The Commissioner is further authorized to charge any fees related to any such extension of time to deposit account 23-3050. This sheet is provided in duplicate.

Date: October 18, 2004

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: **Richard H. Tullis** Confirmation No.: **9155**
Serial No.: **08/078,768** Group Art Unit: **1631**
Filing Date: **June 16, 1993** Examiner: **James Martinell**
For: **Oligonucleotide Therapeutic Agent And Methods Of Making Same**

EXPRESS MAIL LABEL NO.: EL 9985179413 US
DATE OF DEPOSIT: October 18, 2004

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

APPELLANT'S SUBSTITUTE APPEAL BRIEF
PURSUANT TO 37 C.F.R. § 41.37

This substitute brief is being filed in response to the Communication dated September 15, 2004 and in support of Appellant's appeal from the rejections of claims 64-76 and 78-83 dated December 15, 2003. A Notice of Appeal and Request for Oral Hearing were filed April 15, 2004.

1. REAL PARTY IN INTEREST

Based on information supplied by Appellant and to the best of the undersigned's knowledge, the real party in interest in the above-identified patent application is ISIS Pharmaceuticals, Inc., a corporation of Delaware, which is the current assignee.

2. RELATED APPEALS AND INTERFERENCES

There are no other appeals or interferences known to Appellant, Appellant's legal representative, or the assignee that will directly affect or be directly affected by or have a bearing on the Board's decision in the pending Appeal.

3. STATUS OF CLAIMS

Claims 64-76 and 78-83 are pending and rejected in this patent application and are the subject of this Appeal. Claims 64-76 and 78-83 appear in the attached Claims Appendix. Claims 1-63 and 77 have been canceled previously.

4. STATUS OF AMENDMENTS

No amendments to the claims were made in response to the Final Office Action dated September 10, 2002, the finality of which was withdrawn pursuant to 37 C.F.R. § 1.129. In response to the nonfinal Office Action dated June 17, 2003, Appellant filed a Reply pursuant to 37 C.F.R. § 1.111 wherein claim 75 was amended to delete the phrase "at a temperature between 0°C and 80°C" and claim 77 was canceled. The Final Office Action dated December 15, 2003 does not reflect entry of these claim amendments. Appellant is entitled to entry and consideration of the claim amendments pursuant to 37 C.F.R. § 1.129, and they are reflected in the presentation of claims in the attached Claims Appendix. No amendments to the claims have been made subsequent to the Final Office Action dated December 15, 2003. Accordingly, claims 64-76 and 78-83 as amended in the response to the Office Action dated June 17, 2003 are involved in the present Appeal.

5. SUMMARY OF CLAIMED SUBJECT MATTER

Appellant's claimed invention relates to the use of oligonucleotides to specifically inhibit the expression of a target protein in a cell. The invention defined by the pending claims is the product of the surprising discovery made by the Appellant more than twenty years ago that oligonucleotides may be used to regulate protein synthesis in cells via hybridization to nucleic acids. Appellant's discovery provides for the systematic design and use of oligonucleotide agents to specifically block the translation of a target nucleic acid.

In seeking patent protection for his discovery, Appellant presents independent claims 64, 73, 75, 78, and 80. The invention, as recited in independent claim 64, relates to methods for selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids encoding both the target protein and other proteins without inhibiting the expression of other proteins. This can be accomplished by synthesizing an oligonucleotide having a base sequence substantially complementary to a subsequence of a messenger ribonucleic acid encoding the target protein. The oligonucleotide is introduced into the cell where hybridization of the oligonucleotide to the subsequence of the messenger ribonucleic acid occurs to inhibit the expression of the target protein.

Independent claim 73 also recites methods for selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids encoding the target protein and other proteins without inhibiting the expression of the other proteins. According to the method of claim 73, one selects a synthetic oligonucleotide that has enhanced resistance against nuclease enzymes and a base sequence substantially complementary to a subsequence of a messenger ribonucleic acid of the cell encoding the target protein. The synthetic oligonucleotide is introduced into the cell and caused to hybridize with the messenger ribonucleic acid to inhibit the expression of the target protein.

The invention, as defined by claim 75, includes within its scope methods for selective inhibition of the expression of a target protein in a cell producing messenger ribonucleic acids encoding the target protein without inhibiting the expression of the other proteins. One selects a synthetic oligonucleotide having enhanced resistance against nuclease enzymes and a base sequence substantially complementary to a subsequence of a messenger ribonucleic acid of the cell encoding the target protein. The synthetic oligonucleotide is introduced into the cell where it hybridizes to the subsequence of messenger ribonucleic acid.

Claim 78 is directed to methods of selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acid encoding the target protein by selecting a base sequence substantially complementary to the messenger ribonucleic acid of the cell encoding the target protein, providing a synthetic oligonucleotide that is stabilized against *in vivo* degradative enzymes and having the selected base sequence, and introducing the synthetic oligonucleotide into the cell to hybridize to the subsequence of the messenger ribonucleic acid.

As defined by claim 80, Appellant's invention includes methods for selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids encoding the target protein by selecting a plurality of base sequences that are complementary to the messenger ribonucleic acid, providing a synthetic oligonucleotide corresponding to each of the base sequences, selecting a preferred synthetic oligonucleotide for inhibition of the target protein in a cell, and using the selected oligonucleotide to inhibit the target protein in cells.

Appellant's discovery has enabled the development of an array of therapeutic agents that address many previously unmet medical needs.

6. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Appellant respectfully requests review of the adequacy of the Examiner's factually unsupported surmise and speculation to refute the abundant evidence of record demonstrating that the specification and claims as originally filed enable a person of skill in the art to which the invention pertains to make and use the invention commensurate in scope with claims 64-76 and 78-83 without engaging in undue experimentation.

B. As to claim 71 alone, Appellant respectfully requests review of the Examiner's position in the face of Appellant's evidence to the contrary that claim 71 is not patentably distinct over claim 1 of U.S. Patent No. 5,023,243.

7. ARGUMENT

A. The specification and claims as originally filed enable a person of skill in the art to which the invention pertains to make and use the invention without engaging in undue experimentation.

The rejection of claims 64-76 and 78-83 under 35 U.S.C. § 112, first paragraph for alleged lack of enablement is grounded in the Examiner's position that Appellant is required to have expressly taught known forms of stabilized oligonucleotides available at the time of filing in order to enable stabilized oligonucleotides other than phosphotriesters and to have successfully demonstrated inhibition of expression of a target protein using an antisense oligonucleotide *in vivo*. Appellant respectfully disagrees with the Examiner's position. As of the priority date, one of ordinary skill in the art was aware of the existence of stabilized forms of oligonucleotides in addition to phosphotriesters and would have been guided by the

disclosure of the application to the other stabilized oligonucleotides that are suitable, along with phosphotriester oligonucleotides, for *in vivo* use according to the claimed invention. Nothing more than routine experimentation was involved in determining which forms of stabilized oligonucleotides would have worked in the invention. Additionally, unmodified and modified oligonucleotides as used in the present invention are taken up by cells, are sufficiently stable to exert biological activity, and specifically hybridize to the target mRNA, as demonstrated in the examples provided in the specification. The cell culture models exemplified in the specification correlate to *in vivo* biological activity of the stabilized oligonucleotides, as substantiated by pre- and post-filing references. In short, the rejection of claims 64-76 and 78-83 under 35 U.S.C. § 112, first paragraph for alleged lack of enablement should be withdrawn because the evidence of record indicates that those skilled in the art as of October 23, 1981 having the benefit of Appellant's disclosure would have been able to practice the claimed invention without undue experimentation.

Preliminarily, Appellant respectfully asserts that claims 71, 73-76, 78, 79, and 81 are separately patentable from claims 64-70, 72, 80, 82, and 83. Claims 64-70, 72, 80, 82, and 83 are directed to methods of selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids. This is done by hybridizing a synthetic oligonucleotide having a nucleotide sequence substantially complementary to the subsequence of the messenger ribonucleic acid encoding the target protein to the subsequence of the messenger ribonucleic acid to specifically inhibit expression of the target protein. Claims 71, 73-76, 78, 79, and 81 recite methods for selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids. Expression of the target protein is inhibited by a synthetic oligonucleotide having a base sequence substantially complementary to a subsequence of the messenger ribonucleic acid wherein the synthetic oligonucleotide is stabilized to inhibit degradation by nucleases (claims 71, 78, 79, and 81) or has enhanced resistance against nuclease enzymes (claims 73-76).

Because claims 64-70, 72, 80, 82, and 83 do not require enhanced resistance to degradative enzymes or stabilization, in contrast to claims 71, 73-76, 78, 79, and 81, the enablement rejection of claims 64-76 and 78-83 based upon an asserted "problem with uptake and stability of unmodified oligonucleotides" (Office Action mailed June 17, 2003, page 10) is limited to claims 64-70, 72, 80, 82, and 83. Therefore, claims 71, 73-76, 78, 79, and 81 are separately patentable from claims 64-70, 72, 80, 82, and 83.

The enablement requirement of 35 U.S.C. § 112, first paragraph, mandates that the specification teach those skilled in the art how to make and use the claimed invention without undue experimentation. *In re Wands*, 858 F.2d 731, 736-737, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988) (citing *Minerals Separation, Ltd. v. Hyde*, 242 U.S. 261, 270 (1916)). The test of enablement is **not** whether **any** experimentation is necessary, but whether, if experimentation is necessary, it is **undue**. *In re Angstadt*, 537 F.2d 498, 504, 190 U.S.P.Q. 214, 219 (C.C.P.A. 1976). The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *Wands*, 858 F.2d at 737, 8 U.S.P.Q.2d at 1404.

The factors to be considered in determining whether any necessary experimentation is undue include:

i. the breadth of the claims;
ii. the nature of the invention;
iii. the state of the prior art;
iv. the level of one of ordinary skill;
v. the level of predictability in the art;
vi. the amount of direction provided by the inventor;
vii. the existence of working examples; and
viii. the quantity of experimentation needed to make or use the invention based on the content of the disclosure.

Id. (citing *In re Forman*, 230 U.S.P.Q. 546, 547 (Bd. Pat. App. & Int. 1986)). In order to make a rejection, the examiner has the burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 999 F.2d 1557, 1561-62, 27 U.S.P.Q.2d 1510, 1513 (Fed. Cir. 1993). Assuming that sufficient reason for such doubt exists, a rejection for failure to teach how to make and/or use will be proper on that basis. *In re Marzocchi*, 439 F.2d 220, 223-24, 169 U.S.P.Q. 367, 370 (C.C.P.A. 1971). The burden then shifts to the Appellant to provide persuasive arguments, supported by suitable proofs where necessary, that one skilled in the art would be able to make and use the claimed invention using the application as a guide. *In re Brandstadter*, 484 F.2d 1395, 1407, 179 U.S.P.Q. 286, 294 (C.C.P.A. 1973).

The record demonstrates that, as of the time of filing, one of ordinary skill in the art would have been able to make and use the claimed invention using the application as a guide. No undue experimentation was required. Consideration of the *Wands* factors compels a finding of enablement: (1) the breadth of the solicited claims reasonably correlates to the enabled examples using phosphotriester oligonucleotides; (2) numerous modified oligonucleotides were known to one of skill in the art at the time of filing; (3) one of skill in the art of molecular biology in 1981 was highly sophisticated; (4) the specification as filed provided ample guidance, including examples, to one of skill in the art at the time of filing as to how to make and use the invention, which is all that is required for enablement; and (5) nothing more than routine experimentation was required to determine which modified oligonucleotides are most effective in the methods of the invention. Indeed, the record is replete with evidence – facts – underscoring the enablement of the present claims. The Examiner has met these facts only with surmise and skepticism.

It has been asserted that the present invention would not work *in vivo* using double-stranded oligonucleotides. (Office Action mailed June 17, 2003, page 3.) However, there has been no evidence adduced whatsoever for the assertion that a double-stranded oligonucleotide would not work in the claimed methods. This is an immutable requirement for maintenance of the rejection for alleged lack of enablement. The Examiner has merely stated that the term "hybridization" refers to the formation of a double-stranded nucleic acid by annealing of two single-stranded molecules and that the instant application does not disclose the formation of triplex DNA. (Office Action mailed June 17, 2003, page 3.) Appellant has not limited his invention to single-stranded oligonucleotides. Appellant asserts that double-stranded oligonucleotides work in the claimed methods, as supported, for example, by Mercola and Cohen (*Cancer Gene Therapy*, 2(1):47-59, 48-49 (1995)) **(Exhibit 1)**. Disclosure of the mechanism by which double-stranded oligonucleotides work in the invention is not required for enablement. Appellant need only have taught how to make and use the invention without undue experimentation. This Appellant has done.

1. Enablement does not require that Appellant expressly teach known forms of stabilized oligonucleotide available at the time of filing in order to enable stabilized oligonucleotides other than phosphotriesters.

The Examiner maintains that Appellant must have taught forms of stabilized oligonucleotides other than phosphotriesters to have enabled claims 71, 73-76, 78, 79, and 81, the claims requiring stabilization. Appellant disagrees.

Section 112 requires the specification to be enabling only to persons "skilled in the art to which it pertains, or with which it is most nearly connected." *DeGeorge v. Bernier*, 768 F.2d 1318, 1323, 226 U.S.P.Q. 758 (Fed. Cir. 1985). Thus, a patent need not teach, and preferably omits, what is known in the art. *In re Buchner*, 929 F.2d 660, 661, 18 U.S.P.Q.2d 1331, 1332 (Fed. Cir. 1991); *Paperless Accounting, Inc. v. Bay Area Rapid Transit System*, 804, F.2d 859, 864, 231 U.S.P.Q. 649 (Fed. Cir. 1986) ("A patent applicant need not include in the specification that which is already known to and available to the public."); *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986). It has long been the law that a person skilled in the art is deemed to possess not only basic knowledge of the particular art, but also "the knowledge of where to search out information" for section 112 purposes. *In re Howarth*, 654 F.2d 103, 105, 210 U.S.P.Q. 689 (C.C.P.A. 1981).

Nowhere in the specification are the methods of the invention limited to phosphotriester-stabilized oligonucleotides. In fact, throughout the entirety of the specification, it is clearly stated that phosphotriester oligonucleotides are simply one representative example of the stabilized oligonucleotides that may be used in the methods of the invention. For example, the specification states at page 3, "[i]n a presently preferred embodiment of the invention, by way of example and not necessarily by way of limitation, a stabilized oligonucleotide, preferably in a phosphotriester form, is provided . . . " and at page 4, "[t]he preferred oligonucleotide . . . for increased stability, may be transformed to a more stable form, such as a phosphotriester form, to inhibit degradation during use." The application again states at page 5 that the oligonucleotide "can be transformed to a more stable form, such as a phosphotriester form, to inhibit degradation. . . ." Given the language of the specification including "such as," "preferred," and variations thereof, one of ordinary skill in the art readily understands that other forms of stabilized oligonucleotides were contemplated and equally useful in the methods of the invention.

That other forms of stabilized oligonucleotides were known in the art at the time of filing has been firmly established by the declarations of eminent scientists in the field, including Dr. Jerry L. Ruth (August 29, 1994 Declaration **(Exhibit 2)**, Part 5A; April 14, 1995 Declaration **(Exhibit 3)**, Part A), Dr. Dennis E. Schwartz (August 19, 1994 Declaration **(Exhibit 4)**, Part 5A; April 14, 1995 Declaration **(Exhibit 5)**, Part A), and Dr. Stanley T. Crooke **(Exhibit 6)**(Part 5). It is a fact that stabilized oligonucleotides suitable for use in the invention were known and were available to those of ordinary skill in the art in 1981. There is no countervailing evidence of record, none whatsoever.

References of record in this application also support this fact. For example, U.S. Patent No. 3,687,808 to Merigan *et al.* **(Exhibit 7)** describes stabilized phosphorothioate oligonucleotides available as early as 1972 (submitted by way of supplemental information disclosure statement filed June 17, 2002, a courtesy copy of which is submitted herewith). Miller *et al.* (*Biochemistry*, 13(24): 4887-4906 (1974)("Miller 1974")) **(Exhibit 8)**describe the stabilized alkylphosphotriester DNA analogs described in the application. Matzura and Eckstein (*Eur. J. Biochem.*, 3: 448-452 (1968)) **(Exhibit 9)** describe the nuclease resistance of phosphorothioate oligonucleotides. Agarwal and Riftina (*Nuc. Acids Res.*, 6:9, 3009-3024 (1979)) **(Exhibit 10)** show the synthesis of oligonucleotides containing methyl and phenylphosphonate linkages. DeClercq *et al.* (*Virology*, 42:421-428 (1970)) **(Exhibit 11)** set forth the resistance of thiophosphate-substituted oligonucleotides to degradative enzymes. Miller *et al.* (*Biochem.*, 20(7): 1874-1880 (1981)) **(Exhibit 12)** report a stabilized alkyl phosphonate DNA analog having activity *in vitro*. Holy ("Synthesis and Biological Activity of Some Analogues of Nucleic Acids Components," in PHOSPHORUS CHEMISTRY DIRECTED TOWARDS BIOLOGY, W.J. Stec, Ed., Pergamon Press, 53-64, 1980) **(Exhibit 13)** describes modified nucleotide analogs having hydroxyl-containing aliphatic chains that are stable *in vivo* and display inhibitory and substrate activities.

Not only would one having ordinary skill in the art have readily understood that stabilized forms of oligonucleotides in addition to phosphotriesters were contemplated by the invention, but an artisan of ordinary skill also would have known of a number of available stabilized oligonucleotide forms as of the filing date. A routine literature search by an ordinarily skilled artisan at the time of the invention would have yielded a number of available stabilized oligonucleotides suitable for use in the invention, a sampling of which have been provided on the present record. (Crooke Declaration, Parts 3 and 5; April 14, 1995

Declaration of Dr. Schwartz, page 4; April 14, 1995 Declaration of Dr. Ruth, page 4.) As Appellant is not required to teach what is known in the art, his burden has been met.

The enablement requirement does not mandate that the Appellant have presented experiments with each of the available forms of stabilized oligonucleotides to demonstrate that they actually work in the invention. Rather, enablement requires only that Appellant have taught how to determine which stabilized oligonucleotides work in the invention without undue experimentation. Appellant has satisfied this burden by providing representative examples demonstrating his invention. One having ordinary skill in the art need only have substituted for the phosphotriester oligonucleotides of Appellant's examples other known forms of stabilized oligonucleotides to determine their efficacy in the invention. This would not have required undue experimentation on the part of an artisan of ordinary skill.

2. History has proven the naysayers of *in vivo* antisense technology to be incorrect; the present invention was complete and fully enabled in 1981.

The Examiner maintains that the methods of claim 64-76 and 78-83 would not work *in vivo* due to lack of cellular uptake, instability, and unpredictability of hybridization to the target mRNA. The Examiner has placed much reliance on the Gura (*Science*, 270: 575-577 (1995)) **(Exhibit 14)**, Rojanasakul (*Adv. Drug Delivery Revs.*, 18: 115-131 (1996)) **(Exhibit 15)**, and Hijiya (*PNAS USA*, 91:4499-4503 (1994)) **(Exhibit 16)** articles allegedly to show that the present invention is not enabled for *in vivo* use. Appellant asserts that it is improper to base a conclusion of nonenablement upon these few references in view of their actual lack of significance and the numerous other references cited throughout the prosecution of the present application that contradict their allegations. The Declaration of Dr. Sidney M. Hecht **(Exhibit 17)**, a premiere authority in the field of gene expression, supports this assertion.

Dr. Hecht first notes that any concerns raised by the Gura, Rojanasakul, and Hijiya references are directed to the clinical safety of *in vivo* use of antisense technology rather than at the efficacy of *in vivo* antisense methodologies. For example, Rojanasakul at page 118 queries "Can antisense work in living systems?" and responds by stating that while "there are studies which indicate the ***relative safety*** of antisense [oligonucleotides] *in vivo* . . . ***non-specific side effects*** of [antisense oligonucleotides] have also been reported in mice." Rojanasakul goes on to say that these safety concerns "do not diminish the potential use of

[antisense oligonucleotides] *in vivo*, and there are few examples of successful *in vivo* treatment in the absence of specialized delivery systems." (*Id.*) Rojanasakul continues, stating that "[c]onsidering the various obstacles that the antisense [oligonucleotides] must encounter prior to their action . . . ***the desired activity of [antisense oligonucleotides] is observed.***" (*Id.* (emphasis added)). Thus, Rojanasakul actually supports enablement of claims

Likewise, Dr. Hecht notes that Gura avers that "some experts in the field . . . argue that clinical trials have begun far too soon." (Gura at 575.) Dr. Hecht explains that such concerns regarding the clinical safety of antisense oligonucleotides were elicited by the side effects detected in some animal studies. For example, Gura describes one set of experiments in which lethality in monkeys administered a one-time, high-dose injection occurred as well as another set of experiments in which a transient decrease in two kinds of white blood cells and changes in heart rate and blood pressure resulted from the high dose administered. (*Id.* at 576.)

Similarly, the assertion that Hijiya characterizes the field of antisense as being "in its scientific infancy" is misplaced, according to Dr. Hecht. Hijiya makes clear that the unmodified and phosphorothioate-modified antisense oligonucleotides worked therein: "The experiments reported herein serve as a paradigm of [oligodeoxynucleotide]-based therapeutics for human malignancies." (Hijiya at 4503.) Hijiya reasons that, although *MYB* is an effective gene target of antisense oligonucleotides in human melanoma, "further development of the antisense strategy will be needed before the successful application of this technique ***in the clinic*** can be anticipated." (*Id.* (emphasis added).) Appellant likewise asserts that the Mercola reference describes several "signpost" studies in which a reduction in target protein was observed upon *in vivo* administration of antisense phosphorothioate-modified oligonucleotides complementary to the target genes in accordance with the presently claimed methods. (Mercola at 54-55.)

A demonstration of F.D.A. acceptable clinical safety is not required by the first paragraph of 35 U.S.C. § 112. Enablement does not require that the claimed invention satisfy the higher safety standards applied to drugs to be used in clinical trials. According to MPEP § 2107.03, "Office personnel should not impose on applicants the unnecessary burden of providing evidence from human clinical trials.... [I]t is improper for office personnel to request evidence...regarding the degree of effectiveness [in humans] (underlining in

original)." Enablement requires only that the application teach how to make and use the invention without undue experimentation. This requirement has been met: one having ordinary skill in the art would be able to make and use the invention without undue experimentation using only the application as a guide.

Moreover, no drug is free of toxic effects. Fingl and Dixon (Chapter One, "General Principles", In THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 4th edition, L.S. Goodman and A. Gilman, Eds. (1970) **(Exhibit 18)**, courtesy copy enclosed, a copy of which is of record as Exhibit 2 to the Declaration of Dr. Sidney M. Hecht). This fact has been known for many years, as substantiated by Dr. Hecht, and is as true today as it was when first presented in this textbook. For some authors to question the clinical safety of a new drug paradigm is not surprising. If raising such questions were to bar patentability of new drugs, there would be no new drugs. Accordingly, some toxic effects of antisense therapeutics are to be expected. Some expected toxic effects, however, are not an indication that antisense therapeutics do not work *in vivo*.

Indeed, Dr. Hecht attests that any concerns voiced by Gura, Rojanasakul, and Hijiya regarding the use of antisense technology *in vivo* have been proven to be wrong. The successes achieved in the field of antisense technology have been witnessed, thereby ratifying the views of proponents of antisense at the time of the invention and silencing, indeed converting, many critics to what is clearly the correct view: antisense works *in vivo* as taught by the present application.

A number of articles that corroborate the *in vivo* success of antisense technology have been cited during prosecution of the present application. For example, Mirabelli *et al.* (*Anti-Cancer Drug Design*, 6:647-661 (1991) **(Exhibit 19)**, courtesy copy enclosed, a copy of which is of record as Exhibit 3 to the Declaration of Dr. Sidney M. Hecht) notes that antisense oligonucleotides have demonstrated activities against a broad array of targets, that "the therapeutic indexes of phosphorothioate oligonucleotides appear to be quite high," and that "certain phosphorothioates . . . are extremely well tolerated in animals." (Mirabelli at 651.) Mirabelli also provides evidence of successful *in vivo* trials of antisense oligonucleotides. (*See, e.g.,* Mirabelli at 653.)

Crooke (*Ann. Rev. Pharmacol. Toxicol.*, 32:329-376 (1992) ("Crooke 1992") **(Exhibit 20)**, courtesy copy enclosed, a copy of which is of record as Exhibit 4 to the Declaration of Dr. Sidney M. Hecht) corroborates the *in vivo* stability of antisense oligonucleotides, noting

that nuclease activity of sera derived from different species varies, with human being the least active. (*See, e.g.,* Crooke 1992 at 337). Additionally, modified oligonucleotides enter cells at pharmacologically relevant concentrations. (*Id.* at 338-339.) *In vivo* pharmacokinetic studies reveal that antisense oligonucleotides are rapidly and broadly distributed following administration in mice, rabbits, and rats. (*Id.* at 342-343.) Toxicity studies reveal that phosphorothioate oligonucleotides, for example, have high therapeutic indices and exhibit toxicity only at concentrations far in excess of concentrations at which therapeutic activity is observed. (*Id.* at 344; 346-347.) Indeed, Dr. Crooke has attested to these facts on the present record. (Crooke Declaration, Parts 6b and 6c).

Further confirmation of the enablement of Appellant's invention is found in Cossum (*J. Pharm. and Exp. Ther.*, 267(3):1181-1190 (1993) **(Exhibit 21)**, courtesy copy enclosed, a copy of which is of record as Exhibit 5 to the Declaration of Dr. Sidney M. Hecht). That reference describes several *in vivo* studies in which phosphorothioate oligonucleotides were shown to be widely distributed following *in vivo* administration in nothing more than phosphate buffer at physiologic pH. (*See, e.g.,* Cossum at 1181-1182, 1186.) Additionally, Cossum acknowledges that the dosages at which non-antisense effects occur are significantly greater than those at which antisense effects are observed. (*Id.* at 1181.)

Stepkowski *et al.* (*J. Immunol.*, 153:5336-5346 (1994) **(Exhibit 22)**, courtesy copy enclosed, a copy of which is of record as Exhibit 6 to the Declaration of Dr. Sidney M. Hecht) demonstrates specific inhibition of intercellular adhesion molecule-1 (ICAM-1) expression by antisense molecule IP-3082, thereby promoting heart allograft survival. (Stepkowski *et al.* at 5338.) Extension of *in vitro* studies to *in vivo* analyses confirmed the correlation between the efficacy of antisense technology in a Petri dish and in a living organism.

Appellant submits that, not only has the Examiner failed to consider references which run contrary to the few references upon which he relies to assert a lack of enablement, but the Examiner also is relying upon statements that have been proven false. Courts have long and uniformly held that the making of an invention in the face of skepticism by the scientific community is a hallmark of nonobviousness. *Graham v. John Deere Co.*, 383 U.S. 1, 17-18 (1966). Were the Examiner's views on the topic of enablement to prevail, the same skepticism which provides a powerful indication of nonobviousness would simultaneously eviscerate patentability under the enablement standard. This cannot be the law.

It is the Examiner's position that the time lapse between publication of the present application in 1983 and publication of results of success by skilled artisans in an active area of research weighs heavily against enablement of claims 64-76 and 78-83. The time lapse between the effective filing date of the present application and the numerous references cited in support of enablement of claims 64-76 and 78-83, however, has no bearing on the claim that the instant application provides sufficient guidance to one of skill in the art to practice the claimed invention as early as the effective filing date of the instant application. As Dr. Hecht attests, had the pharmaceutical industry in 1981 immediately applied its existing knowledge of medicinal chemistry and pharmacology to the teachings of Appellant, it would have practiced the present invention. Various factors contributed to the lag, not the least of which included establishment, within an organization, of an internal "champion" for a new technology paradigm where the champion is willing to sponsor and defend reallocation of resources from existing programs to a new program. Additionally, once acceptance of the new paradigm is made, established pharmaceutical practice requires pharmacologists to perform substantial and numerous pre-clinical studies to determine the toxicological profile, pharmacokinetics, and pharmacodynamics of any potential drug. Thus, according to Dr. Hecht, it is not unexpected that the generation and reporting of pre-clinical and clinical studies by the pharmaceutical industry related to the efficacy of a potential drug does not immediately follow the publication of the first few positive *in vitro* results.

It has been observed by Fingl and Dixon that "[n]o drug is free of toxic effects." The authors further state, however, that "adverse effects do not arise solely because of the inherent toxicity of drugs and the limitations of the methods for early detection of this toxicity. *Many of the adverse effects could be avoided if drugs were used more carefully and more wisely.*" (*Id.* at 26 (emphasis added).) Further, "[t]he development and evaluation of new drugs in the United States is rigidly controlled by federal regulation administered by the Food and Drug Administration. A new drug may not be marketed for general clinical use until it has been subjected to thorough clinical pharmacological studies and until 'substantial evidence' of its efficacy and safety have been obtained from adequate, well-controlled clinical trials conducted by qualified investigators." (*Id.* at 29.)

Since both positive and negative results must be included in data packages submitted to regulatory agencies, pre-clinical and clinical trials are not performed haphazardly with selective omission of negative results. In other words, slapdash animal studies are not

performed for potential human therapeutic applications because all data collected is subjected to FDA scrutiny. Accordingly, every study is implemented pursuant to highly rigorous standards and carefully planned conditions. Animal tests suitable to regulatory agency submission require established animal colonies and adequate animal care facilities with appropriate veterinary oversight, the development of which is expensive and time-consuming. Accordingly, careful animal experiments do not yield large volumes of publications that appear in the literature quickly. They require systematic studies that may take years to accomplish. In other words, a significant delay in the reporting of pre-clinical or clinical results is entirely routine in the field of drug discovery and development.

Dr. Hecht also affirms that the numerous clinical investigations conducted on *in vivo* antisense methodologies underscore the belief of pharmaceutical companies and, hence, the skilled scientists that comprise them, in the efficacy of antisense technology *in vivo* as detailed by the present application. "Big" pharmaceutical companies became interested in antisense technology after the small pioneer companies confirmed its validity. For example, pioneer companies Hybridon Inc. and Isis Pharmaceuticals, Inc. were incorporated in 1989 for the purpose of developing antisense therapeutics. Gilead Sciences, Inc. formed in 1987 for the same purpose. Genta Inc. was established as a spin-off of Gen-Probe in 1988 with a business objective of developing antisense therapies initiated in Gen-Probe's diagnostic antisense studies. In the mid- and late 1990s, newcomers MethylGene Inc., Inex Pharmaceuticals Corp., and NeoPharma, to name only a few, joined the early-stage companies in exploiting the therapeutic aspects of antisense technology.

In contrast, as explained by Dr. Hecht, while contributing early-published papers regarding *in vitro* related research topics, individual academic researchers, who contribute much of the scientific literature, did not exploit and publish *in vivo* antisense technology. The reasons for this are varied. The exorbitant costs of animal studies, resulting from the necessity of numerous controls as well as the stringent regulations imposed by academic institutions and regulatory agencies, preclude most academic researchers from pursuing such studies absent industrial sponsorship. Additionally, the experiments conducted by most academicians are limited in scope by narrow, well-delineated areas of research interest. Accordingly, academic researchers do not perform isolated experiments that have no bearing on that research interest. Rather, academics are selective in choosing the focus of their experiments, limiting their experimental objectives to the particular area of research that fits

into the grand scheme of the research to which their careers are dedicated, for which they have received institutional approval to study, and for which they have been granted funding.

Dr. Hecht summarizes that antisense technology was developed by small, early stage companies having limited resources. In view of the need of such companies to conserve their limited resources and the knowledge of such companies that a single poorly planned trial yielding a negative outcome could devastate an entire business venture, the pioneer companies in the antisense field had every incentive to perform animal trials carefully and systematically. They conducted animal trials in a highly methodical manner and at timepoints dictated by scientific and business judgment to advance to that phase in the process of moving their drug candidates toward IND status. Pharmaceutical companies, including Isis Pharmaceuticals, Genta Inc., and Hybridon Inc. and their present or past large pharma partners including Novartis, Lilly, Abbott, Merck, Aventis, Amgen, Roche, and Boehringer Ingelheim, have invested huge amounts of time and money to verify the efficacy of antisense drugs in an effort to propel them through clinical phases and into the market. Given the enormous costs associated with drug development and marketing, pharmaceutical companies would not have invested so heavily in the development of antisense technologies if they believed antisense molecules would not work *in vivo*.

Indeed, clinical trials of antisense therapies have definitively established that antisense technology does work *in vivo* in accordance with the principles and guidance set forth in the present application. For example, the antisense drug fomivirsen (Vitravene™; Isis Pharmaceuticals, assignee of the present application) was approved by the FDA for the treatment of cytomegaloviral-induced retinitis in 1998. In practice, administration of fomivirsen, a 21-mer oligonucleotide directed to the major immediate-early transcriptional unit of cytomegalovirus (CMV) is accomplished simply by contacting cells with the oligonucleotide in a pharmaceutically acceptable carrier or diluent such as saline. The FDA-approved mode of administration of fomivirsen for treatment of CMV is intravitreal injection, though any method that would place the relevant cells in contact with the antisense oligonucleotide would work. In short, fomivirsen works *in vivo* in accordance with principles of the presently claimed methods.

The Investigational New Drug Application (IND) for fomivirsen was filed with the FDA in 1993, three years prior to publication of the Rojanasakul reference. Pre-clinical data was included as part of the IND. Thus, prior to the publication of the opinions of skeptics

now relied upon by the Examiner, those skilled in the art already had obtained and submitted *in vivo* data to the FDA, data supporting results contravening that opined by the skeptics. Thus, prior to publication of the negative opinions of skeptics, those skilled in the art had accomplished that which the skeptics opined would not work. In other words, those of skill in the art already were gathering *in vivo* data in support of their IND well before the publication of the opinions of antisense skeptics relied on by the Examiner.

The mere fact that a few naysayers have predicted that methods such as those claimed would not work is of no relevance to the enablement of the instant claims because there is undisputed evidence on the present record that such predictions were incorrect. Some level of skepticism as to advances in science and technology has always been raised, and probably always will. In fact, the magnitude of such skepticism is arguably proportional to the magnitude of the advance. In the final analysis, it is not relevant whether skeptics exist but, rather, whether they were right.

Here, the evidence of record clearly demonstrates that the skeptics that the Examiner has identified were *not* right. The invention as set forth in the application and as presently claimed has been proven to work in the years following the effective filing date of the present application. Indeed, clinical trials of antisense therapies have established that antisense technology does work in accordance with the principles and guidance set forth in the present application, thereby dispelling the criticism of antisense skeptics including Gura and Rojanasakul.

In short, no further disclosure other than that made by Appellant in 1981 was necessary for those skilled in the art to practice the inventions as presently claimed without undue experimentation. This is the hallmark of enablement, and in no way is rebutted by the mere fact that there were those who doubted whether the underlying technology would ultimately be found to work. The methods that Appellant disclosed in 1981 have been demonstrated to work repeatedly thereafter. No clearer case of satisfaction of the enablement requirement of 35 U.S.C. § 112 can be shown.

B. There is no obviousness-type double patenting.

Claim 71 stands rejected under the judicially created doctrine of obviousness-type double patenting as being allegedly unpatentable over claim 1 of U.S. Patent No. 5,023,243 **(Exhibit 23)**. Appellant traverses.

In determining whether a nonstatutory basis exists for a double patenting rejection, the issue is whether any claim in the application defines an invention that is merely an obvious variation of an invention claimed in the patent. When the claimed subject matter is patentably distinct from the subject matter claimed in a commonly owned patent, a double patenting rejection is improper. *Eli Lilly & Co. v. Barr Labs.*, Inc., 58 U.S.P.Q.2d 1865 (Fed. Cir. 2001). Any analysis employed in an obviousness-type double patenting rejection parallels the guidelines for analysis of a 35 U.S.C. § 103 obviousness determination (*In re Braat*, 19 U.S.P.Q.2d 1289 (Fed. Cir. 1991)); however, a double patenting rejection must rely on a comparison of only the claims. MPEP § 804, part III.

Claim 71 recites a method for selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids, the method comprising the steps of synthesizing an oligonucleotide having a base sequence substantially complementary to a subsequence of a messenger ribonucleic acid, wherein the subsequence encodes the target protein, introducing the stabilized oligonucleotide into the cell, and hybridizing the stabilized oligonucleotide to the subsequence of messenger ribonucleic acid to inhibit expression of the target protein.

In contrast, claim 1 of U.S. Patent No. 5,023,243 recites a method of selectively inhibiting *in vivo* synthesis of one or more specific targeted proteins comprising the steps of synthesizing an oligodeoxyribonucleotide having a nucleotide sequence substantially complementary to at least a portion of the base sequence of messenger ribonucleic acid coding for the targeted protein. In the claims of the '243 patent, at least a portion of the oligodeoxyribonucleotide is in the form of a phosphotriester to limit degradation *in vivo*. Claim 1 of the '243 patent calls for introducing the oligonucleotide into the cell and hybridizing the oligonucleotide to the subsequence of messenger ribonucleic acid to substantially block translation of the base sequence and to inhibit synthesis of the targeted protein.

It is asserted that claim 1 of the '243 patent is a specific embodiment of claim 71, thereby rendering present claim 71 obvious. Appellant disagrees. The oligonucleotide of present claim 71 has a sequence substantially complementary to the *coding* portion of the target mRNA, whereas the sequence of the oligodeoxyribonucleotide of claim 1 of the '243 patent is substantially complementary to any region of the mRNA coding for the targeted

protein. Because the portion of the target protein to which the oligonucleotide of claim 1 of the '243 patent is not limited to the coding region of the mRNA, that claim is not a specific embodiment of, and thus does not render obvious, claim 71 of the present application. No *prima facie* case of nonstatutory obviousness-type double patenting exists.

8. APPENDICES

A listing of the claims involved in the present Appeal, as amended by the response filed September 17, 2003, is provided in the attached Claims Appendix.

The following exhibits, already of record as noted, are provided in the attached Evidence Appendix:

Mercola and Cohen (*Cancer Gene Therapy*, 2(1):47-59, 48-49 (1995)) **(Exhibit 1)**, cited in the Reply to Examiner's Answer submitted June 28, 1996 and marked entered by Examiner September 30, 1996;

Dr. Jerry L. Ruth Declaration of August 29, 1994 **(Exhibit 2)**, submitted with the Amendment of August 19, 1994;

Dr. Jerry L. Ruth Declaration of April 14, 1995 **(Exhibit 3)**, submitted with the Amendment of April 14, 1995;

Dr. Dennis E. Schwartz Declaration of August 19, 1994 **(Exhibit 4)**, submitted with the Amendment of August 19, 1994;

Dr. Dennis E. Schwartz Declaration of April 14, 1995 Declaration **(Exhibit 5)**, submitted with the Amendment of April 14, 1995;

Dr. Stanley T. Crooke Declaration **(Exhibit 6)**, submitted with the Response of June 17, 2002;

U.S. Patent No. 3,687,808 to Merigan *et al.* **(Exhibit 7)** (submitted by way of supplemental information disclosure statement filed June 17, 2002);

Miller *et al.* (*Biochemistry*, 13(24): 4887-4906 (1974)) **(Exhibit 8)**, submitted by way of the information disclosure statement of October 15, 1993;

Matzura and Eckstein (*Eur. J. Biochem.*, 3: 448-452 (1968)) **(Exhibit 9)**, submitted by supplemental information disclosure statement of June 17, 2002;

Agarwal and Riftina (*Nuc. Acids Res.*, 6:9, 3009-3024 (1979)) **(Exhibit 10)**, submitted by supplemental information disclosure statement of June 17, 2002;

DeClercq *et al.* (*Virology*, 42:421-428 (1970)) **(Exhibit 11)**, submitted by supplemental information disclosure statement of June 17, 2002;

Miller *et al.* (*Biochem.*, 20(7): 1874-1880 (1981)) **(Exhibit 12)**, submitted by way of the information disclosure statement of October 15, 1993;

Holy ("Synthesis and Biological Activity of Some Analogues of Nucleic Acids Components," in PHOSPHORUS CHEMISTRY DIRECTED TOWARDS BIOLOGY, W.J. Stec, Ed., Pergamon Press, 53-64, 1980) **(Exhibit 13)**, submitted by supplemental information disclosure statement of June 17, 2002;

Gura (*Science*, 270: 575-577 (1995)) **(Exhibit 14)**, cited in the Examiner's Answer of April 30, 1996;

Rojanasakul (*Adv. Drug Delivery Revs.*, 18: 115-131 (1996)) **(Exhibit 15)**, cited in the Examiner's Answer of April 30, 1996;

Hijiya (*PNAS USA*, 91:4499-4503 (1994)) **(Exhibit 16)**, submitted as Exhibit 9 of the Declarations of Dr. Ruth and Dr. Schwartz submitted with the Amendment of April 14, 1995;

Declaration of Dr. Sidney M. Hecht **(Exhibit 17)**, submitted with response of March 7, 2003;

Fingl and Dixon (Chapter One, "General Principles", In THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 4th edition, L.S. Goodman and A. Gilman, Eds. (1970), a copy of which is of record as Exhibit 2 to the Declaration of Dr. Sidney M. Hecht) **(Exhibit 18)**;

Mirabelli *et al.* (*Anti-Cancer Drug Design*, 6:647-661 (1991) **(Exhibit 19)**, a copy of which is of record as Exhibit 3 to the Declaration of Dr. Sidney M. Hecht;

Crooke (*Ann. Rev. Pharmacol. Toxicol.*, 32:329-376 (1992) **(Exhibit 20)**, a copy of which is of record as Exhibit 4 to the Declaration of Dr. Sidney M. Hecht);

Cossum (*J. Pharm. and Exp. Ther.*, 267(3):1181-1190 (1993) **(Exhibit 21)**, a copy of which is of record as Exhibit 5 to the Declaration of Dr. Sidney M. Hecht);

Stepkowski *et al.* (*J. Immunol.*, 153:5336-5346 (1994) **(Exhibit 22)**, a copy of which is of record as Exhibit 6 to the Declaration of Dr. Sidney M. Hecht); and

U.S. Patent No. 5,023,243 **(Exhibit 23)**, first cited by the Appellant to the Examiner in the Response to Advisory Action filed July 20, 1995.

As there are no other appeals or interferences known to Appellant, a Related Proceedings Appendix is not attached hereto.

The Appeal Brief is believed to be fully conforming with rule and statute and is in condition for consideration by the Examiner and response thereto. If the Examiner believes a telephone conference would expedite resolution of the issues on appeal, the undersigned may be contacted at 215-568-3100.

Respectfully submitted,

Date: October 18, 2004

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Enclosures

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: **Richard H. Tullis**
Confirmation No.: **9155**
Serial No.: **08/078,768**
Group Art Unit: 1631
Filing Date: **June 16, 1993**
Examiner: **James Martinell**
For: **Oligonucleotide Therapeutic Agent And Methods Of Making Same**

EXPRESS MAIL LABEL NO.: EL 998517913 US
DATE OF DEPOSIT: October 18, 2004

Mail Stop Appeal-Brief Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

CLAIMS APPENDIX TO APPELLANT'S SUBSTITUTE BRIEF

The following constitutes a complete listing of the claims on appeal. The Amendments of September 17, 2003 in response to the Office Action of June 17, 2003 are included.

64. A method of selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids encoding the target protein and other proteins without inhibiting the expression of the other proteins, said method comprising the steps of:

(a) synthesizing an oligonucleotide having a base sequence substantially complementary to a subsequence of a messenger ribonucleic acid said subsequence coding for the target protein,

(b) introducing the oligonucleotide into the cell; and,

(c) hybridizing the oligonucleotide to the subsequence of the messenger ribonucleic acid to inhibit the expression of the target protein.

65. The method of claim 64 wherein the entire sequence of the oligonucleotide is complementary to the subsequence of a messenger ribonucleic acid coding for the target protein.

66. The method of claim 64 wherein the oligonucleotide is at least 14 bases in length.

67. The method of claim 64 wherein the oligonucleotide is about 23 bases in length.

68. The method of claim 64 wherein the oligonucleotide is between 14 and 23 bases in length.

69. The method of claim 64 wherein the messenger ribonucleic acid is viral.

70. The method of claim 64 wherein the messenger ribonucleic acid encodes a hormone.

71. The method of claim 64 wherein the oligonucleotide is stabilized to inhibit degradation by nucleases.

72. The method of claim 64 wherein the oligonucleotide is an oligodeoxynucleotide.

73. A method of selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids encoding the target protein and other proteins without inhibiting the expression of the other proteins, said method comprising the steps of:

selecting a synthetic oligonucleotide that has enhanced resistance against nuclease enzymes and has a base sequence substantially complementary to a subsequence of a messenger ribonucleic acid of said cell, said subsequence coding for the target protein,

introducing said synthetic oligonucleotide into the cell, and

hybridizing said synthetic oligonucleotide to the subsequence of the messenger ribonucleic acid to inhibit the expression of the target protein.

74. The method of claim 73 wherein said synthetic oligonucleotide is between 14 and about 23 bases in length.

75. **(Previously presented in Amendment submitted September 17, 2003)** A method of selectively inhibiting the expression of a target protein in a cell producing messenger

ribonucleic acids encoding the target protein and other proteins without inhibiting the expression of the other proteins, said method comprising the steps of:

selecting a synthetic oligonucleotide that has enhanced resistance against nuclease enzymes and has a base sequence substantially complementary to a subsequence of a messenger ribonucleic acid of said cell, said subsequence coding for the target protein, and

introducing said synthetic oligonucleotide into the cell to hybridize said synthetic oligonucleotide to the subsequence of the messenger ribonucleic acid.

76. The method of claim 75 wherein said synthetic oligonucleotide is between 14 and about 23 bases in length.

77. **(Canceled in Amendment submitted September 17, 2003)**

78. A method of selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acid encoding the target protein, said method comprising the steps of:

selecting a base sequence substantially complementary to said messenger ribonucleic acid of said cell coding for the target protein,

providing a synthetic oligonucleotide that is stabilized against *in vivo* degradative enzymes, said synthetic oligonucleotide having said selected base sequence, and

introducing said synthetic oligonucleotide into the cell whereby said synthetic stabilized oligonucleotide hybridizes to the subsequence of the messenger ribonucleic acid.

79. The method of claim 78 wherein said synthetic oligonucleotide is between 14 and about 23 bases in length.

80. A method of selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids encoding the target protein, said method comprising the steps of:

selecting a plurality of base sequences that are complementary to said messenger ribonucleic acid,

providing a synthetic oligonucleotide corresponding to each of said base sequences,

selecting a preferred one of said synthetic oligonucleotides for inhibition of the expression of said target protein in a cell, and

using said selected oligonucleotide for inhibition of said target protein in cells.

81. The method of claim 80 wherein said synthetic oligonucleotides are oligonucleotides stabilized against *in vivo* degradative enzymes.

82. The method of claim 80 wherein said selected synthetic oligonucleotide is between 14 and about 23 bases in length.

83. The method of claim 80 further comprising the step of synthesizing bulk amounts of said selected oligonucleotide for inhibition of said target protein *in vivo*.

REVIEW ARTICLE

Antisense approaches to cancer gene therapy

Dan Mercola[1] and Jack S. Cohen[2]

The San Diego Regional Cancer Center,[1] San Diego, California and the Center for Molecular Genetics,[1] University of California, San Diego, California; and Cancer Pharmacology Section,[2] Georgetown University Medical Center, Washington, DC.

Recent advances in the use of oligodeoxynucleotide and plasmid-derived RNA as antisense agents of special relevance to cancer gene therapy are summarized with emphasis on agents and systems which have lead to clinical trials and/or regression of established tumors in animal model systems. Transformed cell lines bearing plasmids and viruses designed for the transcription of antisense RNA have the advantage that they can be characterized thoroughly and the effects of antisense RNA on target gene expression and phenotype can be studied easily *in vivo*. Promising results make the considerable efforts of applying oligodeoxynucleotides in whole animals and in clinical trials more plausible. Conversely, oligodeoxynucleotide experiments which yield promising results in tissue culture can be generalized to the *in vivo* setting by development of clones of cells bearing plasmid-derived antisense RNA against the same target. Several examples of the concordant results for oligodeoxynucleotide and plasmid-derived antisense RNA against the same target are considered. The importance of examination of antisense effects in syngeneic and immunocompetent hosts is illustrated by studies of insulin-like growth factor and insulin-like growth factor receptor where tumor regression and protection against tumor formation have been observed for particular cell types in defined settings.

Key words: ***Antisense RNA; cancer; gene therapy; tumor regression.***

The antisense approach to gene therapy comprises the use of a substance to intervene in the natural processing of genetic information in the cell,[1–3] particularly when an aberrant gene is causing disease. Generally these substances will be nucleic acids, and will contain genetic information in their base sequences. The substance could be produced biologically, ie, by the use of a suitable vector, such as a plasmid or attenuated virus,[4] in the form of an antisense MRNA that binds by complementary Watson-Crick base pairing to the natural sense mRNA. Alternatively, the antisense substance in question will be synthesized chemically. The simplest form of such a "genetic drug" is a small piece of DNA, an oligodeoxynucleotide. These two antisense approaches are shown schematically in Fig 1. A related approach involves ribozymes which are catalytic ribo-oligomers that degrade a complementary mRNA after binding via antisense base pairing (Fig 2).

As well as antisense, there are other related approaches that—instead of inhibiting translation—are designed to inhibit transcription. These are (1) triplex formation, in which an oligomer is targeted to the gene directly and, by binding in the major groove of DNA, forms a triple-stranded helix, and (2) the use of transcription factor decoys that are duplexes designed to bind to a particular transcription factor thereby preventing its normal function (Fig 3). There are many similarities as well as differences between each of these strategies. We will first consider the applications and development of synthetic oligomers, and then the meth-

Accepted January 13, 1995.
Address correspondence and reprint requests to D. Mercola, San Diego Regional Cancer Center, 3099 Science Park Road, Suite 200, San Diego, CA 92121.

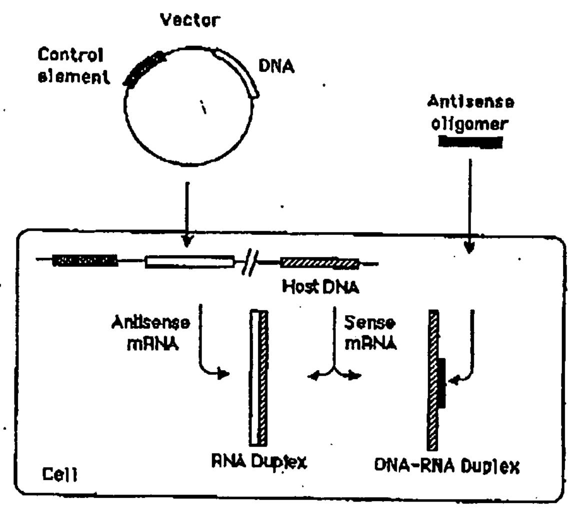

Figure 1. Schematic of translation arrest either endogenously by an antisense mRNA transfected via a suitable vector, or exogenously by an antisense oligodeoxynucleotide.

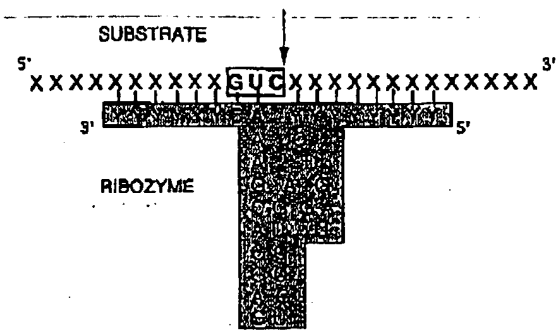

Figure 2. Schematic of a ribozyme; the bases labeled Y are the antisense sequence. The arrow indicates the point of scission on the target mRNA by the active site region.

ods that utilize endogenous antisense mRNA production.

ANTISENSE OLIGODEOXYNUCLEOTIDES

Cell uptake

In all of these genetically based strategies the oligomer used must be able to enter the cell to interact with the genetic machinery, and consequently they must cross the cell membrane. Since these substances are all oligomeric (single or double stranded) and have negatively charged phosphate backbones, their charged nature represents a potential problem. However, there is now ample evidence that these compounds do enter the cell, most likely by endocytosis,[5] and they do reach their target. It should be remembered that since the target (such as mRNA) is present in nanomolar concentrations, only a very small proportion of the extracellular or intracellular drug is needed to be effective. Means to improve the cellular uptake of these substances will be discussed below.

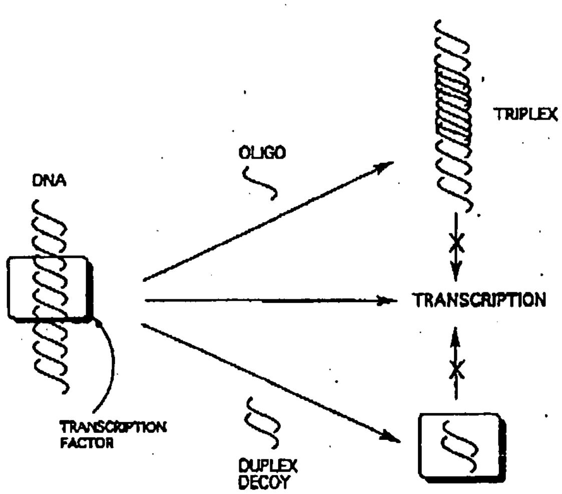

Figure 3. Schematic of transcription arrest either by triplex formation or by transcription factor decoys.

Oligodeoxynucleotide analogs

Cells and organisms protect themselves against foreign DNA and RNA by producing nucleases that degrade phosphodiesters.[6] To transform an oligodeoxynucleotide into an effective drug it is necessary to chemically modify it so that it is resistant to these nucleases. The simplest way of doing this is to modify the phosphodiester backbone, such that a group or atom is substituted for one of the phosphate oxygens, thus forming a resistant bond. The two most common such analogs are the methyl-phosphonate, using a methyl group, or phosphorothioate, in which a sulfur atom is used as a substituent (Fig 4). There are many other ways of modifying the basic mononucleotide unit, and these have recently been reviewed.[3] An interesting new analog is that in which the whole deoxyribose-phosphate backbone is replaced with a peptide-like backbone, to form a series of compounds called the peptide nucleic acids or PNAs (Fig 4).[7] These compounds herald the development of a whole new category of synthetic gene-mimetic substances.[3]

Pharmacokinetics

Several studies of pharmacokinetics of oligodeoxynucleotides have been reported in both mice and rats. Since the phosphorothioate analog is water soluble and is easily synthesized it has been the most widely used and tested for its pharmacokinetic and toxicological properties.[8–10] The results indicate that these oligomers can be expected to provide sufficient tissue concentration to be effective drugs. Notably, the slower plasma clearance process after single dose injection has a half-life of many hours (eg, mean 34 hours in rats[10]).

The Anti-gene approach

Since DNA itself is a tightly bound double helix, it is unlikely that a single-stranded oligomer will be able to disrupt the duplex and bind in an antisense manner. Consequently advantage is taken of the fact that in the B-form of DNA the large major groove leaves enough space for a third strand to bind to form a triple helix or triplex. The specificity for this binding is not Watson-Crick pairing, since the base pair is already formed, but is Hoogsteen (or anti-Hoogsteen) hydrogen-bonded interactions of a third base with the already formed pair.[11] The efficacy of this approach has been demonstrated using catalytic groups, conjugated to the end of an oligomer, that can degrade the target DNA in one (or a few) sites,[12] and it has also been demonstrated to work *in vitro*.[13] However, the development of this approach has not yet proceeded beyond the preclinical stage.

Transcription factor decoys

The idea of sequestering transcription factors, or in general proteins with a binding site for a specific DNA sequence, has been proposed. The use of a duplex of

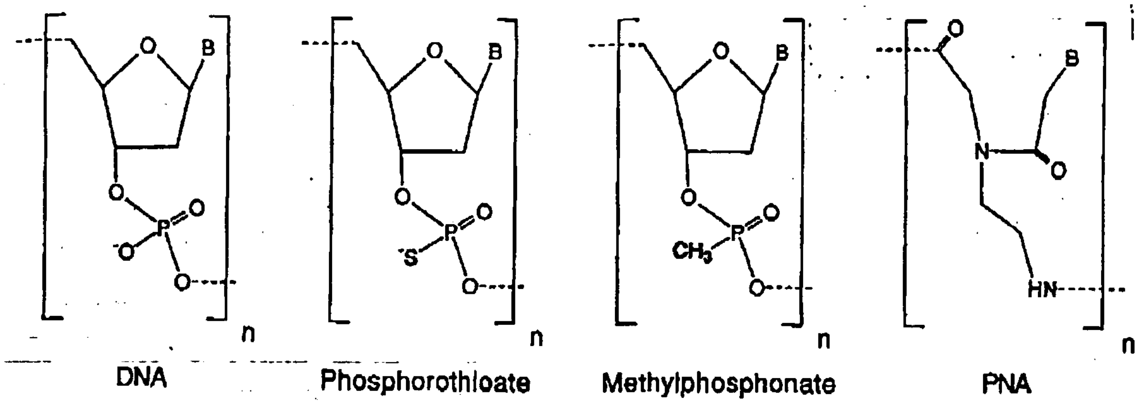

Figure 4. Comparison of monomeric units making up the DNA phosphate backbone structures in natural and "non-hydrolyzable" synthetic drivatives.

phosphorothioate oligomers has been reported.[14] Alternatively, to overcome the problem of degradation by exonucleases, and the potential dissociation of the duplex *in vivo*, cyclic oligonucleotides were developed.[15] These cyclic oligomers have interesting properties, one of which is to bind to a single-stranded region to form a triplex, so they might be useful to select for a single stranded target nucleic acid *in vivo*. However, the non-self–complementary single stranded bridge regions are susceptible to endonuclease degradation. To overcome this cyclic oligonucleotides with nonnucleotide bridges have been synthesized in which the ends are connected 3′ to 5′.[16] These may be effective decoys because they are highly stabilized; they are prepared by ligation after synthesis with the bridges present. This locks the duplex in place and since the bridges prevent unwinding, very high melting temperatures are observed, and the tightly wound helix is also resistant to endonucleases.[16] So far no actual applications of these decoys have been reported.

Antisense

By far the most developed topic in this area of drug development, antisense has the attractive simplicity of an elegant idea. But, in practice, as with any therapeutic modality, problems arise, notably; (a) degradation of the oligomer (even chemically modified analogs are eventually degraded *in vivo*); (b) inefficient cell uptake; (c) nonspecific binding (binding of oligomers to cell membranes and to proteins); (d) nonspecific cleavage of mRNA (due perhaps to partial or transient base pairing of the oligomer to sequences other than the target sequence). Nevertheless, some 36 applications of phosphorothioate oligonucleotides have been reported,[3] and these represent a considerable body of evidence that antisense is a useful approach for delineation of gene function, apart from its therapeutic potential.

Ribozymes

Ribozymes are essentially antisense oligomers in which the antisense portion flanks an RNA active site that cleaves a bound RNA. Ribozymes exist naturally, but have been adapted to degrade a specific mRNA sequence.[17] Note that although ribozymes are generally considered to be composed entirely of RNA, it is possible to synthesize analogs in which many of the residues are chemically modified, for example, with 2′ substituents such as O-Methyl and Fluoro.[18] Recently a clinical trial has begun of a ribozyme targeted on HIV.[19]

Methods of delivery

Apart from direct injection of oligomers, several methods of delivery have been tried *in vitro*, by forming complexes with positively charged lipids,[20] by incorporation into liposomes,[5] and by electroporation. At present no single method has been reported that combines the properties of both efficacy and targeting. Two parameters may be helpful, (a) the fact that oligomers tend to accumulate in specific tissues,[8] thus providing a crude tissue targeting, and (b) the use of sterically hindered immunoliposomes used to deliver genes in gene therapy, may also become carriers for oligomers. It should be noted that oligomers are almost certainly protein-bound *in vivo*, and the impact of this fact on their efficacy, delivery, and excretion has not been clarified. Specific examples of systemic delivery to block or promote repression are discussed in comparison with the results for the corresponding stably produced antisense RNA in transfected clonal lines in the next section.

Clinical trials

The ability to carry out clinical trials of oligonucleotides represents the accumulation of several years of effort, not only preparing oligodeoxynucleotides and analogs in large quantities,[21] but also raising this synthesis to the level of good manufacturing practice. As an example of the use of an antisense oligonucleotide in an animal disease model, an anti-c-myb phosphorothioate oligonucleotide has been reported to result in the elongation of life of *nu/nu* athymic mice in a model tumor system.[22] Clinical trials of antisense oligonucleotides are underway against five diseases (Table 1).[22-26] Overall several

Table 1. Clinical Trials of Antisense Phosphorothioate Oligonucleotides

Disease	Target*	Company†
AIDS	HIV	Hybridon, Inc.
Chronic myelogenous leukemia (CML)	myb BCR/ABL	Lynx Therapeutics, Inc.
Acute lymphoblastic leukemia (AML)	p53	Lynx Therapeutics, Inc.
Genital warts	HPV	Isis Pharmaceuticals, Inc.
Retinitis	CMV	Isis Pharmaceuticals, Inc.

*HIV, human immunodeficiency virus; HPV, human papillomavirus; CMV, cytomegalovirus.

†For further information contact the company.

hundred patients have been treated in these trials, and so far no untoward side effects have been reported. Because of the cost of these compounds, local or topical application is preferred, for example in the use against genital warts the drug is injected at the site of the lesion,[25] and for retinitis it is used in drops.[26] In the cases of the leukemias, bone marrow purging is another strategy[27] that could maintain efficacy while keeping costs down. In the case of HIV-1, at least one ribozyme that cleaves HIV-1 RNA is in phase I clinical trials and the GEM91 phosphorothioate oligodeoxynucleotide of Hybridon, Inc which targets *gag* RNA transcript sequences, is starting phase II trials in France (Table 1).

The use of antisense oligonucleotides to treat AML illustrates the versatility as well as the vicissitudes experienced during the development of antisense p53 oligonucleotides for clinical use (E. Bayever, personal communication). The tumor suppressor gene product, p53, is—surprisingly—markedly elevated in all AML patients studied. p53 is rarely elevated in nonmanipulated normal bone marrow cells and rarely mutated in AML. The findings that p53 is an abnormally elevated protein were the basis of cell culture studies which revealed that antisense oligonucleotides targeting p53 caused efficient cell killing. However, kinetic studies revealed that, although p53 levels decreased initially in antisense treated cells, the levels returned to normal or higher values by 72 hours. The elevated levels persisted for days suggesting that apoptosis may account for the impressive *in vitro* killing.

A phase I clinical trial employing graded doses of an antisense p53 oligonucleotide failed to exhibit evidence of therapeutic effects.[23] The *in vivo* setting differs from that in tissue culture in that the oxygen *in vivo* level is much reduced. Tissue culture experiments were carried out at low-oxygen tension and no effect of the p53 oligonuleotide was seen. The condition of increased cellular oxygen was modeled by adding agents which promote "oxygen stress" such as H_2O_2 and Mitoxantrone[91]—a known AML chemotherapeutic agent. The use of oxygen radical producers was associated with increased the levels of p53—possibly owing to DNA damage. These conditions restored the effect of antisense p53 oligonucleotides at low-oxygen tension. Phase I/II clinical trials[42] are currently underway in which a combined approach of IV infusion of 0.20 mg of synthetic phosphorothioate antisense p53 oligonucleotide/kg of body weight per hour for 7 days followed at 48 hours, the optimum time for enhancement, by treatment with Mitoxantrone for 3 days and cytosine arabinoside for 5 days. The complete course will last for 7 days.

In a separate clinical trial, *ex vivo* bone marrow purging is also being explored using the same antisense p53 reagent but at somewhat reduced levels.[42] Marrow cells are exposed to 10 μM oligonucleotide once for 36 hours before reinfusion. The antisense treatment is combined with pretreatment using conventional chemotherapeutic agents such as VP-16, BCNU and others several days before reinfusion with the "purged" marrow. To date nine patients have been treated.

Prospects

The therapeutic application of oligonucleotides and their derivatives is very much in its infancy. While the initial expectations of a few short years ago may not have been realized in immediate cures, this unlikely outcome rested more on concepts of science fiction and unrestrained hope. The fact that there are five clinical trials of antisense oligomers underway, and that there are many start-up companies working intensely on their own specialized approaches, indicates that the hard problems that need to be overcome in transforming a simple idea into a truly therapeutic method are being tackled. It is to be hoped that human diseases of genetic origin will eventually yield to approaches based on these genetic medicines.

RECENT ADVANCES IN PLASMID-DERIVED ANTISENSE RNA

Oligonucleotide versus plasmid delivery

The use of antisense RNA producing constructs for the inhibition of transformation has been growing steadily (Fig 3) since the application of Izant and Weintraub in 1985 to inhibit the synthesis of actin.[28] There are important advantages and disadvantages compared with oligodeoxynucleotides. First, oligodeoxynucleotides likely function first in the cytoplasm to effect arrest of translation, direct ribonuclease-H activity and to inhibit crucial functions by selective protein binding as well as other effects. Plasmid-derived RNA is produced in the nucleus. Nuclear hybrid formation with target mRNA may interfere with target mRNA transport from the nucleus, may stimulate modification and breakdown of the mRNA *via* ribonuclease H-like activity, may interfere with transcription of the complementary DNA strand and may bind exposed regulatory DNA sequences. Second, plasmid-derived antisense RNA is usually very large compared with a typical 15-20 nt oligodeoxynucleotide and, therefore, may have more than one binding site on the target mRNA. Also, the large size may favor the formation of nonspecific interations. In addition, antisense RNA and/or mRNA may

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have considerable secondary structure which may interfere in hybrid formation.

The major advantages of stably expressed antisense RNA derives from the ability to generate stable cell lines for thorough analysis. Mass cultures or cloned cell lines with stably inserted DNA which encodes antisense RNA allow for thorough examination of a broader range of properties including the *in vivo* phenotype as observed in syngeneic or immunodeficient recipients. For example, in the case of antisense RNA directed against insulin-like growth factor (IGF-I), *in vivo* studies lead to the observation of an apparent enhanced immune response which was associated with complete tumor regression.[51] Once a crucial target or desirable effect has been identified, the justification for the extensive investment necessary for *in vivo* studies with oligodeoxynucleotides directed against the same target is more plausible. Conversely, promising results that were originally observed with antisense oligodeoxynucleotides in tissue culture may be more easily extended to *in vivo* setting for tumor inhibition studies by development of antisense RNA-expressing clones.

If a relatively large segment of DNA has been used for stable production of antisense RNA, it may be necessary to carry out a deletion analysis to identify the most active sequence. Holt et al[34,35] employed a relatively short 84 bp segment of 5′ noncoding *c-fos* DNA in antisense orientation to successfully inhibit *c-fos* expression *in vivo* and Schönthal et al[63] used as little as 40 nts of 5′ *c-jun* in antisense orientation to successfully inhibit *c-jun* expression. Finally, if retroviral or other viral constructs are used, the option is available to create retroviruses or other infective agents for application *in vivo*.[65-66]

Direct targeting of causative oncogenes

Amini et al[30] first used antisense *src* RNA expressing plasmids to inhibit the expression of v-*src* in *src*-transformed cells which substantially reduced tumor formation in athymic mice. Thus, direct targeting of the causative oncogene may have efficacy in a tumor formation model (Table 2). This has been extended to human tumor cells using techniques that are promising for therapy.[45,73] The human large cell lung carcinoma cell line H460a, there is a diallelic mutation in K-*ras* at codon 61, a change leading to an activated K-*ras* and transforming product. The K-*ras* mRNA was targeted by infection of the cells with a retrovirus of very high titer (amphotropic-ecotropic "ping-pong" reinfection protocol) which contained 2 kb of K-*ras* cDNA bearing exons II and III in sense or antisense orientation with respect to a β-actin promoter. Up to 95% of the tumor cells could be transduced following 5 to 7 exposures to the retrovirus. Remarkably, antisense RNA expressing clones, but not sense controls, exhibited specific suppression of K-*ras* steady-state transcript and protein levels with little decrease in other Ras family members even though there is up to 85% sequence identity (N-*ras*, exon II). The antisense-regulated cells exhibited suppressed growth in soft agar. Tumor formation and tumor growth in athymic mice was much reduced—but not eliminated (Table 2). The experiments have been extended to a clinical trial.[42]

Antisense N-*ras* has been applied to human pLC/prf/5 hepatoma lines by direct infection with an amphotrophic retrovirus.[81] Human lung diploid fibroblasts and sense control hepatoma cells were unaffected while the antisense-expressing cells were severely growth retarded.

Antisense H-*ras* combined with a hammerhead ribozyme has been exploited to inhibit several tumorigenic human cell lines (Table 2). Stable lines of human bladder carcinoma EJ cells bearing the ribozyme were implanted in the bladders of athymic mice.[84] Tumor take was considerably reduced and subsequent invasion was decreased leading to an almost twofold increase in survival compared with mice bearing tumors of control cells. The results clearly indicate the potential of ribozymes as anticancer agents.

Antisense oligonucleotides and plasmid-derived antisense RNA have been used to evaluate the role of CRIPTO, a member of the epidermal growth factor receptor family, in the transformation of human GEO and CBS colon carcinoma cells.[36] Two different antisense phosphorothioate oligodeoxynucleotides suppressed CRIPTO expression and growth but had no effect on CRIPTO-negative colon carcinoma cells. Clones infected by a retrovirus bearing CRIPTO sequences in antisense orientation also exhibited reduced CRIPTO expression. When transplanted into athymic mice, tumors of the antisense regulated cells appeared later and grew slower than the CRIPTO-negative clones (Table 2).

A recurring theme is the inability to completely suppress tumor formation (Table 2) and, by implication, an inability to suppress established tumors. As one possibility, the presence of multiple oncogenic contributions, which are common in aneuploid human tumors,[40] may be a complication. Thus, transcription factors such as *c-fos* which act downstream of several known protooncogene products such as c-*src*, c-*ras*, or c-*raf* and multiple growth factor signal transduction pathways have been targeted (Table 2). Antisense *c-fos* RNA completely eliminated *c-fos* expression in v-*sis* transformed cells which continuously express a potent mitogenic factor, PDGF-B/v-*sis*, which has over 90% homology to the PDGF-B/c-*sis* product.[32] The antisense-regulated cells exhibited a flat morphology, restored contact inhibition as well as decreased tumorigenicity in athymic mice.[32] Other studies targeting *c-fos* in *ras*-transformed cells yielded qualitatively similar results (Table 2). However, *complete* suppression of tumor formation has not, in general, been achieved (Table 2). The instability of vectors *in vivo*, the presence of multiple mechanisms of immortalization and transformation may all contribute to the problem. Advances have been achieved with the use of antisense RNA-directed against components of the insulin-like growth factor-I (IGF-I) signal transduction pathway of glioblastoma cells.[37,50-51,53-56] The results provide hints for optimizing other systems such as those targeting *c-fos* and related genes.

Table 2. Examples of the Use of Plasmid-Derived Antisense RNA for Control Tumorigenicity

Construct	Gene Target	Transduced Cell Type	Animal Model	Remarks	Ref.
pMMT1srcINV Inducible MT prom. 3.6 kb AS	c-src	MTAg-transformed FR3T3 Cells	Syngeneic Rats; TUMORIGENICITY REDUCED	Early use of plasmids for tumorigenicity control.	30
pSVsof; 1.7 kb antisense w. exons I & II	c-fos	PDGF-B/sis-transformed HIH-3T3 cells	Athymic mice; TUMORIGENICITY REDUCED	c-fos is required for PDGF-B/ serum stimulation of cell division.	31–32
pMMTVsof dex. inducible; 84 bp antisense		H-ras-transformed	TUMORIGENICITY REDUCED	Suppression of anchorage-independent growth.	35
C-Ha-Ras AS 2 kb w. exon I	c-H-ras	H-ras-transformed GCM-3T3 and REF-4.3 cells	Athymic mice; TUMORIGENICITY REDUCED	Observed reduced lung met frequency by GCM-3T3.	39
βACTIN-K-rasAS/ LNCX 2 kb AS w. exons I & II	c-K-ras	human lung carcinoma H460a	Athymic mice; TUMORIGENICITY REDUCED	Specific suppression of specific Ras family member: activated K-ras.	45
EJpHβH-rasRB Hammerhead Ribozyme	c-H-ras	H-ras-transformed NIH-3T3 cells; MCF-7 breast carc.	COMPLETE INHIBITION OF TUMORIGENICITY	Catalytically inactive antisense RNA control plasmid resulted in decreased tumorigenicity only.	49
		Hu bladder carcinoma EJ cells	Athymic mice; REDUCED TUMOR TAKE REDUCED INVASION	Athymic mice with bladder implants studied. Ribz treated cells associated w. 2× survival.	84
pSV1-ASuPA-265	Urokinase	murine melanoma B16-F10 cells		Transfection of B16-F10 cells with human urokinase expression vect cause enhanced metastases which is reversed by antisense.	46
pR509-8-18 CMV prom. 1 kb AS	BCL2	BCL/c-myc transformed human Jurkat T-cells	Athymic mice; BLOCKED TUMOR FORMATION	Scid mice exhibiting a CML model (BV173 cells) greatly reduces BCR-ABL mRNA *in vivo*.[43]	44
pMSG021, dexamethasone inducible	p120	p120-transformed NIH-3T3 cells; MCF-7 hu breast carc.	Athymic mice; TUMORIGENICITY REDUCED	Application of antisense to human tumor line *in vivo*.	47
Retrovirus 900 nt AS hygromycin resistance	CRIPTO EGF receptor family member	Hu Colon carcinoma GEO & CBS cells	Athymic mice; REDUCED TUMORIGENICITY	Infection of control WIDR cells w/o CRIPTO receptor did not alter growth.	36
pANTI-IGF-I episomal replication via expression of EBNA & EB ori; MT prom., 1 kb AS	IFG-I	Rat glioblastoma C6	Syngeneic rats; COMPLETE INHIBITION OF TUMORIGENICITY; REGRESSION OF ESTABLISHED BRAIN TUMORS	Antitumor effects involve glioma-specific CD8+ lymphocytes; antisense IGF-I RNA may block "tolerance" mechanism; human clinical trial protocol approved 6/93.	50–51
pHSP-IGF-IR 309 nt AS	IGF-I Receptor	Rat glioblastoma C6 Hu glioblastoma T98G	Syngeneic rats (C6 only); COMPLETE INHIBITION OF TUMORIGENICITY; CHALLENGE REJECTION; REGRESSION OF ESTABLISHED TUMORS	Conforms Antisense IGF-I results; PDGF-B induces IGF-I Receptor expression in human T98G glioma cells; Construct blocks autonomous and anchor. indep. growth of T98G cells.	37 54

Abbreviations: AS, antisense; Prom., promoter; scid, severe combined immunodeficient mouse model; Hu, human; MT, metallothionein gene promoter; MMTV, mammary tumor virus promoter; MTAg, Middle-T antigen; LNCX, retroviral vector with L = LTR of murine Moloney leukemia virus, N = neomycin phosphotransferase gene, C = human cytomegalic virus promoter and X = arbitrary insert of choice all in the order given; EB, Epstein-Barr virus; EBNA, EB nuclear-associated protein; EGF, epidermal growth factor; IGF, insulin-like growth factor; Ref., reference (see REFERENCES).

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IGF-I and the control of tumor growth

For some fibroblast lines it has been possible to demonstrate that certain growth factors act in a sequential fashion (for a review see ref 29). PDGF preferentially acts on events of G_0/G_1 of the cell cycle to recruit quiescent cells into the cell cycle and to effect a state of "competence" to respond to "progression" factors.[28,33–34] Progression factors act in late G_1 to allow cells to cross a checkpoint and become committed to S phase. For many cells, such as rat C6 and human T98G glioblastoma cells, IGF-I is a major progression factor. Thus, for tumors that continuously express PDGF, IGF-I, and the corresponding receptors, it would be expected that intervention in these systems may inhibit tumor growth. For example, cells transformed by the SV40 large T-antigen (LTAg) often exhibit a reduced requirement for any of the growth factors in serum.[57] However, primary mouse fibroblasts derived from "knockout" mice with a homozygous disruption of IGF-I receptor (IGF-IR) gene can not be transformed by LTAg. Transformation can be restored by cotransfection of the "knockout" cells with both and source of LTAg and IGF-IR. These experiments convincingly demonstrate the dependence of transformation by LTAg on the IGF-I/IGF-IR autocrine system for mouse fibroblasts.

IGF-I and IGF-IR are not expressed in normal adult glial cells, but are an important components during the development of the central nervous system.[58] However, IGF-IR appears to be commonly expressed in gliomas.[59–62] The significance of targeting the IGF-I autocrine system in neurological tumors is suggested by the recent experience of J. and J. Ilan and coworkers[50–51] who targeted the IGF-I gene in rat C6 glioblastoma cells and by Baserga and associates[37] who targeted IGF-IR in rat C6 and human T98G glioblastoma cells. The former studies also reveal a striking role of the immune system.

For the IGF-I antisense studies, stable lines of the rat C6 glioblastoma cells were developed by transduction with a plasmid containing the Epstein-Barr virus origin of replication and encoded nuclear antigen I. This construct also contains an IGF-I coding sequence in antisense orientation with respect to a metallothionein promoter thereby providing inducible antisense RNA expression.[50–51] The cells exhibited metal-dependent inhibition of IGF-I expression and repression of the transformed phenotype in tissue culture. Antisense RNA-expressing cells failed to develop tumors in immunocompetent and syngeneic rats following doses of 10^7 cells whereas parental and control cells bearing an irrelevant plasmid invariably developed tumors within 2 weeks. Similar results were observed for B-104 neuroblastoma cells bearing the antisense RNA expressing vector.[50] The tumorigenicity of neuroblastoma cells which do not express IGF-I were not prevented by expression of antisense IGF-1 RNA and the regression of isolated tumors was not effected by treatment with such cells.

Inflammatory nodules containing numerous mononuclear cells were often noted at the site of injection of the antisense RNA-producing tumor cells. This was taken as evidence of an enhanced immune response to the antisense RNA-producing cells.[50–51] The affect could be harnessed to promote regression of established tumors both at subcutaneous and intracranial sites. First, parental C6 glioblastoma cells, which rapidly form grossly detectable tumors 4 to 6 days after a single injection of 10^7 cells, were implanted in one hind limb. Following the appearance of tumors 4 to 6 days later, antisense RNA-producing cells or parental cells were injected into the contralateral limb. Although sample sizes were small (n = 10), all tumors including those of control and parental cells in animals bearing the antisense RNA-producing cells regressed within three weeks and these animals remained tumor-free for 13 months.[51] Second, protection against recurrence was shown. If established subcutaneous tumors were surgically removed, a high proportion (5 of 6) developed local recurrences. However, of the surgically treated animals that had been inoculated after surgery with 10^7 antisense RNA-producing cells, none of six treated animals developed recurrences.

The most clinically relevant animal model of glioblastoma is the case of an established intracranial tumor in an immunocompetent and syngeneic host. Remarkably, when this condition was modeled by intracranial inoculation with parental C6 tumor cells followed by an inoculation at a systemic site with 10^7 antisense RNA-producing cells, none of the six animals developed an intracranial tumor after 4 months whereas five of five control animals which had not received a systemic inoculation died or showed clear evidence of tumor formation.[51]

Several caveats are apparent such as the small sample sizes. Also, it is unclear how large the intracranial implants were at the time of the systemic inoculations which were carried out 2 to 3 days after the intracranial implantation injection. Several separate experiments utilizing a variety of controls showed that the antisense RNA-producing cells acted as an efficient vaccine against the development of peripheral tumors. For example, rats injected at two sites with either parental glioma cells or control glioma cells that had been transfected with the vector devoid of IGF-I DNA, invariably developed tumors whereas similarly treated animals injected at one of the two sites with the antisense RNA-producing cells were completely protected against development of a tumor at the contralateral site. Similar results, including the involvement of $CD8^+$ mononuclear cells at the sites of tumor regression have been reproduced in a separate mouse model based on totipotent PCC3 embryonal carcinoma cells.[52]

IGF-1 Receptors and the control of tumor growth

Recently, the essential aspects of the results with IGF-1 have been reproduced in both C6 cells and extended to human T98G glioblastoma cells, by targeting IGF-1R.[37,54] The approach has the advantage of inhibiting additional factors which work through IGF-1R such as IGF-2. Stable antisense IGF-1R-producing cells were

unable to make tumors in syngeneic rats whereas wild-type-C6 cells developed tumors within a week. The antisense RNA-producing cells provided protection against a subsequent challenge of wild type C6 cells. Moreover, inoculation of wild type C6 tumor-bearing rats with antisense RNA-producing cells lead to complete regression of the established tumors in all 18 rats within 2 weeks. The role of inflammation during the tumor regression period was not assessed.

The experiments have been extended to human T98G glioblastoma cells.[54] These cells continuously express IGF-1. They also continuously express PDGF-B/c-sis (unpublished observations), a factor which induces the production of IGF-1R in T98G cells and "primes" these receptors leading to an enhanced mitogenic effect by IGF-1.[57] Indeed, these cells are growth arrested in the absence of serum but exhibit serum-independent growth following transfection with an IGF-1R expression vector thereby apparently bypassing the need for serum and/or PDGF-B.[54] Moreover, transfection with a vector expressing IGF-1R antisense RNA blocks growth induction by PDGF and the ability to form colonies in soft agar. Thus, the autocrine interaction of IGF-1 with its receptor appears to regulate anchorage-independent growth in these human tumor cells. Unfortunately, T98G cells are poorly tumorigenic[59] and the elegant animal modeling observed with the C6 system may be very difficult to carry out.

The regulation of growth of glioblastoma cells is complex and involves other systems than PDGF and IGF such as basic fibroblast growth factor that is likely an important autocrine factor,[82] suppressor gene products[83] and others which may have to be taken account of for a complete understanding.

A role of IGF-1R and IGF-II in human breast cancer has recently been described,[48] suggesting that the significance of the IGF-autocrine system may not be confined to neural tumors.

Immunocompetent host response

The relationship between IGF-1 and immunity is intriguing. The cellular infiltrates accompanying tumor regression were predominately composed of $CD8^+$ lymphocytes consistent with a specific immunological response as opposed to a nonspecific chronic inflammatory response. Moreover, lethally irradiated (5000 rads, ^{60}Co) antisense RNA-producing cells also acted like a true vaccine in that the animals inoculated with the irradiated cells were protected against tumor formation by a subsequent challenge throughout the period of observation, over 12 months.[51] The basis for the $CD8^+$ response is not known, however, it has been suggested that the reexpression of IGF-1, a fetal and neonatal growth and differentiation factor, by the tumor cells serves to reestablish a phenotype which includes evasion of immune recognition.[51–52] Other explanations such as the antisense-mediated cell death which may allow the release of additional cellular antigens, the possible role of antisense RNA as an antigen or as an adjuvant or the possible role of altered histocompatibility antigens and other possibilities need to be evaluated. Nevertheless, the potential of antisense IGF-1/IGF-1R-producing cells in treatment is evident. A phase I clinical trial for human brain tumors using *ex vivo* transduced antisense IGF-1 RNA-expressing autologous tumor cells as a vaccine has been approved and is due to start in December of 1994.[42]

Complete tumor regression has also been observed for several systems using so-called suicide genes which convert a prodrug into a cytotoxic agent.[85] Tumor regression appears to come about, in part, owing to the "bystander effect" which may extend lethality of suicide genes to cells that have not been transduced by the sensitizing constructs. A number of explanations have been advanced and utilize the proximity of untransduced cells to the transduced cells.[85] In addition, immune mechasnisms involving both $CD4^+$ T helper and $CD8^+$ T suppressor/cytotoxic lymphocytes have been implicated.[85] Recently, evidence was presented that tumor regression also can occur in preexisting control-cell tumors in the same animals and is accompanied by the infiltration with inflammatory cells which secrete a variety of cytokines such as TNF, Il-1, Il-6, IFN-gamma, and GM-CSF.[87] These observations, together with the observations of antisense IGF-1, raise the prospect that the sudden killing of even a portion of tumor cells may, in some settings, sensitize the host. It will be of interest to learn whether similar immunological responses of an immunocompetent host are invoked by different mechanisms of sudden cell killing.

The *in vivo* *delivery problem*

Methods for the transduction of a high percentage of target cells for gene replacement or for the treatment of established tumors are deficient at present. Thus, stable antisense RNA expression systems may have their greatest worth in identifying targets that would be useful to control *in vivo* by use of oligodeoxynucleotides. Two questions arise. Are oligodeoxynucleotides a feasible means of delivering an antisense-based compound and is there concordance between the results for antisense oligodeoxynucleotides and plasmid-derived antisense RNA? Human and animals trials of oligodeoxynucleotides are in their infancy and comparisons among approaches are sparse, however, several signposts are available.

Local delivery. Neckers and colleagues[64] have explored the use of implanted "minipumps" to achieve high local steady doses of oligodeoxynucleotides. Antisense and sense oligodeoxynucleotides of the N-myc sequence were delivered to the vicinity of subcutaneous tumors in athymic mice. The treatment lead to an average 50% tumor reduction in the antisense treated animals whereas there was no regression in the control animals. Over 70% of the tumors exhibited regression whereas irrelevant oligodeoxynucleotides had no effect.

Systemic delivery. The first intravenous application of antisense oligodeoxynucleotides to an animal model was explored by Wickstrom et al[70] who observed a 50% decrease in c-Myc protein in peripheral lymphocytes 3 to

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4 hours following a 300 nmol IV bolus of phosphorothioate oligodeoxynucleotide complementary to the c-*myc* sequence. The effects of sustained systemic application of antisense oligodeoxynucleotides in an animal model was studied by Ratajczak et al (*cf* Table I).[22] Severe combined immune deficient (scid) mice were used to establish a model of human leukemia by implantation of K562 cells. Four weeks later the animals were treated with phosphorothioate oligonucleotides complementary to human c-*myb* by steady infusion from a subcutaneous "minipump" providing 100 g of oligonucleotide/d (~15 nmol) for up to 14 days. All animals eventually succumbed to leukemia; however, the animals treated with scrambled or sense oligodeoxynucleotides did not survive longer than the saline-controls whereas the antisense treated animals lived on average 3.5 times longer or about 5 to 6 weeks from the onset of treatment.

Higgins et al[38] have explored both plasmid-derived antisense RNA and systemically delivered oligodeoxynucleotides to target the p65 subunit of the transcription factor NFKappaB. NFKappaB may be a crucial regulator of multiple genes that encode molecules which are essential for cell adhesion such as integrins and cell adhesion molecules. Direct targeting of integrins by retrovirally introduced antisense integrin RNA inhibits neuroblast migration in developing chick embryos and leads to disruption of integrin-mediated interaction and loss of cell numbers. Inhibition of this system in transformed cells may block essential structural features of stromal attachment thereby blocking tumor formation. (*cf* ref 85). Thus, stable lines of *ras*-transformed Balb/c-3T3 cells bearing a dexamethasone-inducible construct for antisense RNA production exhibited a dexamethasone-dependent inhibition of p65 mRNA production as monitored by RT-PCR and failed to grow in soft agar. The sense-bearing control lines were unaffected by dexamethasone. Moreover, examples of significant regression of established tumors were described. Tumors of several antisense RNA expressing clones were first allowed to develop for 2 to 3 weeks before addition of dexamethasone to the drinking water. Dexamethasone treatment did not inhibit tumor growth in the control sense-injected animals whereas a uniformly smaller tumor size was described for the antisense RNA expressing cases. In summary, as for the studies of CRIPTO,[36] concordance between RNA and oligodeoxynucleotide experiments was observed.

For the oligodeoxynucleotide experiments, 18-24 nt phosphorothioates complementary to the 5′ region including the translation start site were used. The oligodeoxynucleotides were introduced into athymic mice by either twice weekly injections (average 0.4 mg/d) or by continuous infusion by means of a "minipump" (average 0.2 mg/d) [~25 nmol]. Five × 10^6 K-balb fibrosarcoma or B-16 melanoma cells were injected 72 hours after the start of treatment. Over 70% of the antisense-treated animals were judged to show a clear reduction in tumor size compared with saline, sense, or irrelevant oligodeoxynucleotide controls. Analysis of the remaining tumor mass showed that p65 mRNA was preferentially reduced in the antisense-treated cases and that NfKappaB binding activity was significantly reduced in nuclear extracts from tumors derived from antisense-treated animals compared with sense-controls. Interestingly, extensive separate studies[68] of the toxicity of the *sense* oligodeoxynucleotides, showed considerable toxicity at somewhat higher doses than those used for the tumor studies.

Bolus injections may have some effect (refs 67,70; T. Ochiya, personal communication). Phosphorothioates complementary to FGF4 RNA were used to study limb bud development in an organ culture system where marked inhibition was observed compared with olionucleotides with scrambled sequences or sense oligodeoxynucleotides. Moreover, FGF4 transforms NIH-3T3 cells and the phenotype was specifically inhibited in culture by 500 nmol/L solutions of the same antisense oligonucleotide. Based on this experience, the antisense oligodeoxynucleotides were then applied to athymic mouse model by intravenous injection of a 50 g-liposome complex 3 days after subcutaneous inoculation of 10^7 tumor cells (Ochdiya, personal communication). Animals treated with sense or scrambled-sequence oligodeoxynucleotides developed tumors which were 14 mm in diameter after 8 to 10 days whereas the antisense treated animals exhibited nodules of 2 mm or less. No information on the distribution or stability of the complex *in vivo* is currently available.

Future

A number of technologies are on the horizon which may have a significant impact. For example, lipsome and lipid-complexed antisense oligodeoxynucleotides is an area of intense activity, eg, refs 20, 71–72.

Recombinase and integrase-related proteins have been used to facilitate ribozyme function through enhanced strand exchange.[75–77] These novel methods employ the *Escherichia coli* RecA enzyme or other recombinases to catalzye the formation of homologous complexes between short seqments of single- or double-stranded "probe" DNA with homologous sites of target DNA. Probe DNA is precoated with RecA which facilitates the search for and interaction with homologous target DNA to form stable "displacement loops" or "D-loops." Target DNA could be crucial regulatory sequences of promoters for oncogenic proteins. The formation of stable "D-loops" also means that continuous supply or expression of antisense DNA or RNA may not be necessary. Examples of this form of inhibition include blocking viral promoters or the insertion of a 33 nt DNA segment to block the GC-rich Sp-1 binding site.[88] Recently, these methods have been extended to targeting the p53 gene in native chromatin of metabolically active isolated nuclei suspended in agarose.[90] These techniques have potental application in both controling expression and in homologous replacement of altered genes (D. Zarling, personal communication; for a review see ref 88).

DNA- and RNA-targeting may be augmented by the development of proteins and peptides with DNA- and

RNA-binding properties of arbitrary specifity.[78-79] Combinational display libraries of DNA-binding proteins following randomization of the DNA-contact sites of the zinc-finger protein Egr-1 have been developed.[78-79] The display library may be screened and the clones with the desired specificity may be isolated and cloned by various panning procedures. Interestingly, at least one Zinc-finger-bearing protein, native Egr-1 itself, has been shown to inhibit the transformed phenotype including tumorigenicity of PDGF-B/v-*sis*-transformed NIH-3T3 cells[80] and in at least one human tumor cell line.[53,80] Deletion studies show that the effect, indeed, resides in the 300 nt fragment encoding the DNA-binding zinc-finger domain.[80] The new randomization approaches hold promise for a vastly extended range of interactions.

Single chain recombinant antibodies specific for oncogenic products such as ErbB-2 have been cloned from hybridoma cells and stably expressed in human cancer cell lines such as ovarian carcinoma and shown to efficiently block the expression of target protein and proliferation (D. Curiel, personal communication). A variety of controls show that expression of a specific and high affinity single-chain antibody occurs and leads to down regulation of cell surface ErbB-2 (D. Curiel, personal communication). This sophisticated technique requires the preparation of cDNA encoding regions of two chains of the normal monoclonal antibody combining site. While not an antisense technique, the approach targets translated products and may be seen as complementary.

Finally, an obvious extension of the antisense approach to targeting two or more oncogenes thought to be contributing to the transformed phenotype has not been systematically explored although simultaneous and significant reduction of two tomato gene products has been reported by stable expression of chimeric antisense RNA[91] providing some validity to the concept. Thus, a number of intriguing techniques are being developed which separately or in combination may prove useful for intervention.

Summary

The use of expression systems for the formation of stable cell lines provides a system for thoroughly characterizing the effect of antisense RNA on the target and cell line of choice including the effects *in vivo*. *In vivo* studies with such cell lines provide a means to examine tumor formation, the ability to promote regression of established tumors and may, as in the case of IGF-I, provide insights into new mechanisms. Moreover, it may be of considerable interest to examine other targets and to reexamine previous systems employing antisense to c-*fos* and related systems such as antisense to PDGF in immunocompetent hosts. The information learned from studies of stable lines may justify the large effort involved in preparing and delivering oligodeoxynucleotides *in vivo*. Although information to date is limited, in the few systems where the effects of oligodeoxynucleotides in tissue culture have been compared with the stable expression of much larger antisense RNA complementary to the same target, concordance has been observed. However, in no case has hybrid formation between antisense RNA and target RNA been shown to be the basis of an antitumor effect *in vivo*. Numerous controls will be necessary to establish that a specific antisense mechanism is at work (*cf* 69). Although it is early days, modified oligodeoxynucleotides, local delivery systems such as depots and time release devices appear to be possible methods for optimization. Improvements in viral vectors[93] to allow greater tissue specificity and transduction efficiency may supersede this "two-step" approach but until that desirable time, a serviceable pattern for animal studies may be emerging. Finally, emerging novel techniques may significantly enhance the future armory.

ACKNOWLEDGEMENTS

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231, on Aug. 29, 1994

By Jo Ann Horiuchi Dallas

PATENT

Attorney Docket No. 16243-1-5

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Richard H. Tullis	Examiner: J. Martinell
Serial No.: 08/078,767	Art Unit: 1805
Filed: June 16, 1993	DECLARATION PURSUANT TO 37 C.F.R. § 1.132
For: OLIGONUCLEOTIDE THERAPEUTIC AGENT AND METHODS OF MAKING SAME	

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

I, Dr. Jerry L. Ruth, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true and statements made on information or belief are believed to be true. The Exhibits (1-8) attached hereto are incorporated herein by reference.

2. I received a Ph.D. in synthetic organic chemistry from the University of California at Davis in 1978.

A copy of my curriculum vitae is attached as Exhibit 1.

3. I am presently employed at the U.S. Fish and Wildlife Forensics Laboratory where I am primarily responsible for conducting DNA analysis to assist in the identification of wildlife for threatened and endangered species of animals. Prior to my obtaining employment at the Forensics Laboratory, I was a vice president of research at Molecular Biosystems where my responsibilities involved the development of nucleic acid based diagnostic kits.

4. I have read and am familiar with the contents of the application and related papers. I understand that the Examiner has made two rejections. The first rejection is for failure to fully teach how to make and use the invention as claimed, and the second is that the invention is obvious over a combination of four references. This declaration will address both rejections. I will provide objective evidence of the state of the art of arresting specific protein expression by oligonucleotide hybridization in 1981. The evidence will be provided by interpretation of references relating to this art and by my personal perspective from having been involved in the field of oligonucleotide chemistry and biology in 1981.

5. **ENABLEMENT**

A. Analogs of Nucleic Acid

It is my understanding that the Examiner has concerns that the invention should be limited to phosphotriester-modified nucleic acid and that claims reading on natural nucleic acid and other analogs are not enabled. It is my opinion that once Dr. Tullis identified functional sizes and the mRNA coding region as a target, the invention was fully disclosed to one of skill. The utility of natural nucleic acid and various analogs to be internalized by cells and inhibit cell function was known. Evidence of these facts can be found in the prior art. For example, Miller (1977) describes neutral, nonionic nucleic acids for nonspecific inhibition of protein synthesis. Befort (1974) used methylated ribonucleic acid to inhibit viral replication. Zamecnik and Stephenson (1978) used a natural phosphodiester DNA tridecamer to inhibit viral replication *in vivo*. Finally, Summerton (1979) described a number of early reports using various nucleic acid analogs to inhibit viral infections (see page 89).

B. Making Ribonucleic Acid

The Examiner further argues that there is no teaching of how to make or use ribonucleotides. The methodology for making synthetic RNA and its analogs was available in 1981. Evidence of this fact can be found in the Miller reference (1977). Therein the authors used an analog of ribonucleic acid to non-specifically inhibit protein synthesis.

Moreover, methods for chemical synthesis of oligoribonucleotides were well established by 1981 as is illustrated by reference to the work of Ohtsuka

and his colleagues. In the mid and late 1970s, Ohtsuka's group reported synthesis of numerous oligoribonucleotides corresponding to the sequence of an *E. coli* tRNA. Some of this work is described in *Nuc. Acids. Res. Symp. Series (NARS)* No. 7, pp. 335-343 (1980), which is attached as Exhibit 3, and the cites therein. In 1980 Ohtsuka reported synthesis of oligonucleotides corresponding to the total sequence of *Escherichia coli* $tRNA_f^{met}$; these oligos were joined using RNA ligase to create an entirely synthetic tRNA (*Id.*). The synthesis of *E. coli* tRNA is also discussed in a subsequent paper that appeared prior to the filing date of the application [Proc. Nat. Acad. Sci. 78(9) 5493 (1981); attached as Exhibit 4]. Clearly, by 1981 methods for chemical synthesis had been available for several years.

Enzymatic methods for synthesis of oligoribonucleotides complement chemical methods and include use of polynucleotide phosphorylase and T4 RNA ligase. RNA ligase in particular has been useful in synthesis of oligoribonucleotides and in 1980 Gumport *et al.*, in a paper on T4 RNA ligase, observed that "...the enzyme is now widely used to synthesize defined sequences of RNA." [NARS No. 7 (1980) pp. 167-171 at 167; attached as Exhibit 5]. In a 1981 review attached as Exhibit 6, ("T4 RNA Ligase as a Nucleic Acid Synthesis and Modification Reagent" in *Gene Amplification and Analysis*, Vol. 2, Chirikjian and Papas, eds. Elsevier (New York) 1981, pp. 314-345 at pages 335-339), Gumport and Uhlenbeck describe work by several groups engaged in oligoribonucleotide synthesis, including Ohtsuka [described above], Neilson and colleagues [using a combination of organic and enzymatic methods to prepare several decanucleotides], Krug and colleagues [preparation of a 21-nucleotide RNA], and others [see citations at 337, first full paragraph]. In my opinion, the attached exhibits clearly demonstrate that methods for synthesis of oligoribonucleotides were well known in 1981.

C. <u>Cell Uptake of Nucleic Acid</u>

Finally the Examiner raised the issue of cell uptake of nucleic acid. He comments that there are no data and methods for actually "getting short DNAs or RNAs into cells." Living cells have a natural capacity for internalizing short nucleic acids. There is nothing to teach. The cells internalize these compounds without

special culture conditions. There is no need to render the cells porous. The literature relied upon by the Examiner to support his obviousness rejection teaches this fundamental fact. For example, the Miller reference involves the effect of a trinucleotide analog on mammalian cells and the Summerton reference discloses at pages 93-94 the routine uptake by animal cells of both RNA and DNA. Finally there is the paper by Zamecnik and Stephenson (1978) which describes the internalization of viral infected cells by a DNA of 13 nucleotides.

6. OBVIOUSNESS

It is my understanding that the Examiner believes that in 1981 a person of skill reading Itakura *et al.*, Paterson *et al.* or Hastie *et al.* and Summerton or Miller *et al.* would have had a motivation and a reasonable expectation that targeting the coding region of a specific mRNA with a oligonucleotide complementary to the coding region would have arrested protein translation of that mRNA. There are a number of objective reasons why this is not an accurate statement of the state of the art in 1981.

A. The secondary structure of the mRNA made it an unlikely target for control of expression by complementary oligonucleotides.

The claimed invention went against the conventional wisdom of the time. The conventional wisdom in 1981 was that the secondary structure of mRNA was extensive that there was no reasonable likelihood that a oligonucleotide complementary to a coding sequence would have sufficient access to arrest translation inside a living cell. In addition, those of skill understood that the natural mechanism of peptide elongation by ribosomes involved the destabilization of the extensive secondary structure of the mRNA. For these reasons, the idea of hybridizing a complementary oligonucleotide to a coding region of mRNA to arrest translation was contrary to conventional wisdom. The oligonucleotide would have to overcome two significant hurdles. First it had to bind to the coding region of the mRNA, which was viewed as a Gordian knot of secondary structure. And even if the complementary oligonucleotide could find and anneal to its complementary subsequence, the ribosomes were viewed as able to read mRNA coding regions constrained by extensive secondary structure. Thus it was not likely that the

hybridization of a complementary oligonucleotide would arrest translation. The ribosome would simply "toss" the duplexing structure aside.

Although there is little express language in the prior art articulating the above concerns, the literature indirectly compels one to conclude that secondary structure and ribosomal helix destabilization were concepts teaching away from the invention. References published after the priority date of the subject application do expressly identify the state of the art at the time of the invention.

The extensive secondary structure of mRNA was well understood in 1981. In the book, The Ribonucleic Acids, 1977, Eds. Stewart and Latham, Springer Verlag, Chpt. 4, "Messenger RNA" by J.M. Adams, the extensive secondary structure in phage mRNA and eukaryotic mRNA is described. Those of skill recognized the importance of mRNA secondary structure and particularly were aware of the need of low mRNA secondary structure in the regions where ribosomes initially bind to mRNA. W. Salser, in his chapter Globin mRNA Sequences: Analysis of Base Pairing and Evolutionary Implications, in *Chromatin* Vol XLII (1978) pages 985-1001 at page 992, provided a graphic illustration of the extensive amount of secondary structure in the typical mRNA. In Dr. Salser's illustration, the sequences involved in ribosome initiation and interaction are dramatically free of secondary structure. Dr. Adams, on page 103 in his section (e) entitled "Influence of secondary and tertiary structure on initiation," also discusses the importance of secondary structure on initiation of translation. In chapter 7, by Wettenhall and Clarke-Walker of *The Ribonucleic Acids*, the authors on page 258 in a section entitled "Messenger RNA structure" expressly state, "[t]he role of mRNA structure in initiation reactions is receiving considerable attention." These authors also discuss the role of secondary structure of mRNA on translation efficiency.

The concern over availability of the coding region to bind oligonucleotides is made more apparent when one looks at the secondary references relied upon by the Examiner. These references are Paterson *et al.* and Hastie *et al.* describing cell-free, *in vitro* experiments in which denaturing conditions to relax the secondary structure of their mRNA are applied prior to hybridizing nucleic acids. For example, Patterson used 100°C for 30 seconds and

Hastie used temperatures between 45°C and 65°C. For the Examiner to believe that one of skill would have understood that complementary oligonucleotides were able to bind to the coding regions of mRNA under *in vivo* conditions when both Hastie and Paterson used denaturing conditions is logically inconsistent and scientifically incorrect.

A number of other references taught that the targeting of a coding regions would not be a preferred target for a oligonucleotide agent expected to control expression. Pluskal *et al. Biochem. Soc. Trans. 7*:1091-1093 (1979), wrote that their work with a heterogenous mixture of low molecular weight oligonucleotides non-specifically inhibited translation, but that the mode of action was not affected by preincubation with the mRNA. One of skill is left to conclude that the translation inhibition observed by Pluskal did not occur via complementary binding interactions between the oligonucleotides and the mRNA.

Continuing in temporal order, the Ts'o patent further suggests away from the Applicant's invention. In the Ts'o patent, the claimed oligonucleotide analogues are described as inhibiting expression of a preselected sequence, either a cellular nucleic acid or a viral nucleic acid. Ts'o uses trimer analogues and at column 25, lines 5-10, they state that their analogues inhibited poly(U) messages but not globin messages. In column 26, lines 5-34, Ts'o explains that they saw no inhibition *in vivo* of either bacteria or hamster cells.

Ts'o's results are consistent with later published reports. These later reports actually explain what was intuitively apparent to those of ordinary skill at the time Dr. Tullis filed his application in 1981. There was no objective reason or basis to conclude that the coding region of mRNA was an effective target for *in vivo* control of expression by trinucleotides because there was no evidence that the trimers used by Ts'o inhibited elongation when bound to mRNA either *in vitro* or *in vivo*. Either the coding regions of mRNA were physically unavailable to complementary oligonucleotides or even if binding to the coding regions, the oligonucleotides were unable to arrest the elongation process of the ribosomes. As explained above, the latter concept was particularly compelling because to elongate, ribosomes have to untwist (denature) the secondary structure of mRNA.

Perhaps the most express and compelling articulation of the true state of the art from 1977 until after the effective filing date of the subject application is found in Dr. Miller's own work published in two parallel reports in 1985. In Blake, Murakami and Miller, *Biochemistry 24*, 6132-6138 and *24*, 6139-6145, the authors discuss at length their concerns over secondary structure on the availability of subsequences of mRNA to complementary oligonucleotides. In the first of the two companion articles (A22), Dr. Miller looked at 8-12 mer oligonucleotides and concluded, on page 6135, that in the cell free rabbit reticulocyte protein expression system, 8-mer oligonucleotides do not arrest protein elongation, but that if bound to the initiation codon region the oligonucleotides will arrest translation. On the second column of page 6136, Dr. Miller in summarizing the state of the art expressly states:

> The above results are in agreement with the recent findings of Liebhaber *et al.* (1984). They found that cDNAs to human globin mRNA which cover the initiation codon or extend into the 5'-noncoding region are able to completely inhibit translation in a rabbit reticulocyte lysate. In contrast cDNAs which cover only the coding region exclusive of the initiation site are not effective at blocking translation. These authors postulate that a helix-destabilizing activity associated with the reticulocyte ribosomes is able to disrupt secondary structure during the elongation step but not the initiation step of translation. **Thus, cDNAs or oligonucleotides bound to the coding region of mRNA would be expected to be unable to prevent translation in the reticulocyte system.** [Emphasis added]

Dr. Miller is summarizing two points to explain why oligonucleotides binding to the initiation codons will inhibit translation but those binding to coding regions will not inhibit translation. He first explains that the ribosomes will simply remove the oligonucleotides bound to the translated region (page 6136, column 2), and second, he explains that the secondary structure of mRNA precludes the binding of the oligonucleotides to the translated region (page 6137). He implies this is true even for cDNAs which may be hundreds of bases long.

In the second article, Dr. Miller is again reporting on the ability of oligonucleotides to arrest *in vitro* translation. In this report the oligonucleotides are between 6 and 11 bases long. The authors are reporting some degree of success with the binding of oligonucleotides to mRNA coding regions. Although the

success of the method is being reported, the authors are clearly articulating the concerns over secondary structure which was the conventional wisdom in 1981. For example at page 6144, the authors state:

> In addition to the effect of mRNA secondary structure, the region of the mRNA to which the oligonucleotide methylphosphonates binds influences its effectiveness as an inhibitor of mRNA translation. The results in Table II suggest that oligomers complementary to the 5' end and initiation-codon regions are somewhat better inhibitors than the oligomers which bind to the coding regions. **Thus it appears that the initiation step of translation is more sensitive to oligomer mRNA binding than the elongation step of translation.** [Emphasis added]

Finally, as late as 1986, the literature was still suggesting that short oligonucleotides will only arrest translation if bound to the initiation sites. In particular, Haeuptle *et al.,* using a cell free translation system, clearly states that size of the oligonucleotides bound to coding regions is a critical key to ensuring arrest of translation. Just as is taught by the subject application with its effective filing date of 1981, Haeuptle reports in 1986 that the oligonucleotides must be 10 bases or greater in length before substantial inhibition of translation is demonstrated. The Examiner should also note that the authors, in deference to secondary structure, are using 55°C for 5 minutes to relax the mRNA sufficiently to allow the oligonucleotides to bind to their target regions.

In conclusion, I state that the conventional wisdom in the art, at the time of the invention, taught away from Dr. Tullis' invention. The literature cited above is offered as evidence of this wisdom. This literature clearly teaches that the secondary structure of mRNA, especially in the coding region, was thought by those of skill in 1981 to be extensive and that this secondary structure would preclude binding of a complementary oligonucleotide to the coding region mRNA for the purpose of arresting translation. Also, there was reason to believe that the helix destabilization of ribosomes during elongation would have easily displaced any oligonucleotide duplexed on the coding region. Therefore, one of skill would not have considered the claimed invention to have a reasonable expectation of success in 1981 when Dr. Tullis originally filed the subject patent application.

B. <u>The Examiner's interpretation of the text in Miller (1977) goes well beyond the understanding one of skill would have reading the same text in 1981.</u>

It is my further understanding that the Examiner has stated that Miller (1977) expressly states that oligonucleotides complementary to the coding region of mRNA might inhibit cellular protein synthesis *in vivo*. The two statements have been cited by the Examiner. The abstract states:

> Our biochemical studies suggest that inhibition of cellular protein synthesis might be expected if $G^mp(Et)G^mp(Et)U$, $G^mp(Et)G^mpU$, and G^mpG^mpU, which have been taken up by or formed within the cell, physically bind to tRNA and mRNA and inhibit the function of these nucleic acids.

The last paragraph states:

> The triester $G^mp(Et)G^mp(Et)U$ is complementary to target nucleic acids involved in cellular protein synthesis and has a transitory effect on the cells which is then relieved by its degradation. Such a transitory effect of inhibition may provide a convenient and useful way of imposing a temporary interruption of cellular functions. On the other hand, neutral oligonucleotide analogues with longer chain lengths and a greater resistance to hydrolysis would be expected to achieve a greater specificity and effectiveness in regulating the function of target nucleic acids inside the cell over a long period of time. The observations of the present study lay the groundwork for continued investigation into the use of neutral oligonucleotide analogues as proves and regulators of nucleic acid function within living cells.

There are multiple reasons why one of skill in 1981 would not have interpreted the cited text in the way the Examiner suggests. The text clearly does not state that oligonucleotide analogs could bind to the coding regions of mRNA. At best it is ambiguous when taken out of context both literally and historically. It is literally ambiguous because the authors' work is directed to general inhibition of all protein expression by using a trimer to bind to the amino acid accepting codon of a tRNA. There is no evidence of trimer binding to target mRNA or of any inhibition of specific protein expression due to complementary binding of Miller's trimer to mRNA.

The phrase "greater specificity" is patently ambiguous. Statistically, a trimer sequence is represented every 64 nucleotides and therefore a timer binds

non-specifically. Miller's use of "greater specificity" could refer to oligonucleotides that are specific only for the amino acid accepting codon of tRNA or that will bind specifically (only) to the initiation region of a mRNA, or thirdly, as the Examiner would read the passage, using longer oligonucleotides that would bind with greater specificity to the coding region of an mRNA.

In historical context, the meaning of the above quoted text is clearly directed to either the tRNA or mRNA binding sites. These were only regions perceived by scientists in 1981 as being sufficiently accessible to complementary oligonucleotide binding under *in vivo* conditions. As explained above, the extensive secondary structure of mRNA was a primary reason why those of skill would have avoided the coding region of mRNA to arrest translation. For example, in Dr. Salser's review article of 1978, he includes a figure (Figure 3) depicting the proposed secondary structure of a mRNA. It is an extraordinary complex and twisted structure. Only a few regions are accessible to a complementary oligonucleotide and these are designated as points of initiation and tRNA interactions. These open regions which are generally found as single-strand hairpin loops.

Furthermore, the historical record indicates that by April of 1981, Dr. Mill understood the limitations of his 1977 report. In *Biochemistry*, 20:1874-1880, Exhibit 8, Dr. Miller *et al.* reported on the arrest of globin expression via blockage of tRNA aminoacylation by trimers and tetramers. On page 1879, and at Table VI, they report no effect on globin synthesis despite the fact that the oligonucleotides used by Miller (polyA) could bind to at least three sites in the coding region of mRNA encoding globin (codons 45, 85 and 118). See Exhibit 9.

Finally, in 1985, the last two Miller references summarize the historical evidence that taught away from targeting the coding regions of mRNA with oligonucleotide to arrest translation. First, in the introductions to both 1985 papers, there is no reference to the 1977 paper when discussing mRNA as a target for control of protein expression by complementary oligonucleotides. Secondly, as stated above on pages 7-8, the 1985 references specifically suggest; (1) that secondary structure will prevent binding of oligonucleotides to mRNA; and, (2) that helix destabilizing properties of ribosomes will remove oligonucleotide even it they

were to have access to the coding region of an mRNA. These are two conclusive reasons why one of skill would not expect the arrest of protein translation by oligonucleotide binding to the two conclusive coding regions of mRNA.

In summary, by 1981, Miller's reference to "greater specificity" would not have been interpreted as a suggestion for controlling the expression of particular "target" proteins by binding to specific coding regions of mRNA, but as a suggestion to use longer oligonucleotides to bind **specifically** to the open regions of the tRNA or mRNA that bind to rRNA during the initiation step of synthesis. The open binding sites are longer than three bases and thus one would expect greater specificity for binding by using oligonucleotides greater than three bases. For these reasons, I state definitively that one of skill reading Miller in 1981 would have recognized that the proposed targets for binding oligonucleotides are these binding sites.

To argue that in 1981, one of ordinary skill would have predicted *in vivo* utility for an oligonucleotide binding to the coding portions of mRNA is to read Miller in a vacuum, and ignores the authors' data and the historical understanding of the accessibility of the coding region of mRNA due to secondary structure.

C. <u>Although the Examiner reads the Miller reference as suggesting the use of an oligonucleotide binding to the coding region of an mRNA, Miller does not suggest this aspect of Dr. Tullis' invention.</u>

The Miller reference is silent as to the target sequences on a mRNA to which its oligonucleotides might bind. Messenger RNAs have several functional domains. The coding region is only one domain. These various domains are described as "signals" by W. Salser in his chapter Globin mRNA Sequences: Analysis of Base Pairing and Evolutionary Implications, in *Chromatin* Vol XLII (1978) pages 985-1001. The signal sequences are described on page 988 as including "ribosome binding, mRNA processing, transport from the nucleus to the cytoplasm and so on".

Dr. Tullis' claims involve only the coding region and one reading Miller would not have been directed to this region. One of skill with knowledge of the secondary constraints of the coding region of mRNA and the mechanism by which

ribosomes read mRNA would not have read Miller as suggesting the targeting of the mRNA coding region.

D. Even if the prior art oligonucleotides of Miller were binding to the coding region of an mRNA, they could not have arrested translation.

As further evidence that the Examiner's interpretation of Miller goes beyond any reasonable interpretation of the reference by one of skill in 1981, I would like to point out that the inhibition detected by Miller was in fact solely due to a non-specific interference of tRNA. We know this because Miller uses only trimer oligonucleotides and trimers were later shown to be useless as inhibitors of translation even if bound to a coding region of an mRNA.

The Examiner is directed to the 1986 reference of Haeuptle *et al.* Haeuptle describes the *in vitro* arrest of translation by oligodeoxyribonucleotides. The authors relaxed the secondary structure of mRNA encoding lysozyme using 55°C and hybridized oligonucleotides of varying lengths to a portion of the coding region. On page 1435 and in Figure 4 on page 1439, they present evidence of their inability to arrest translation using short oligonucleotides of 5 bases. The 5-mer species are simply too small to have sufficient binding strength to arrest the progress of a ribosome. The authors reported successful arrest of translation with their 10, 15 and 20 mer species.

E. The Examiner has misinterpreted the use of "specificity" by Miller in 1977.

The Examiner relies on the final paragraph of Miller, 1977 stating: "... oligonucleotide analogs with longer chain lengths and a greater resistance to hydrolysis would be expected to achieve a greater *specificity* and effectiveness in regulating the function of target nucleic acids...". The Examiner reads this sentence to include a suggestion to use oligonucleotides for longer binding to the coding regions of mRNA. Above I have explained that this phrase is and would have been understood by one of skill in 1981 to be directed to the non-coding regions of mRNA. I would like to focus this part of my declaration on evidence that the authors' reference to "specificity" was in a general context and did not refer to the coding regions of mRNA encoding specific proteins. More particularly,

the authors were referring to oligonucleotides that bound more specifically to the amino acid accepting end of the tRNA and the initiation sites of mRNA.

Dr. Miller's later publication, Blake *et al.* (1985A) provides evidence of his intended meaning in using "specificity" in 1977. On page 6137, column 2 is the word "specific[1]" used in the same context that the word "specificity[2]" was used in 1977. Miller is again asserting that his work suggests *specific* control of expression by binding oligonucleotides to mRNA. But the context of the 1985 reference clearly implies that the arresting oligonucleotides are not directed to the coding regions of the mRNA. The Blake article states that the only available regions are the initiation points due to secondary structure. Thus the Examiner's interpretation of the text of Miller (1977) in an unlimited and broad context so as to include the coding regions of mRNA is to ignore the scientific realities of the contemporaneous literature.

F. <u>There are objective scientific reasons why one of skill would have not had a reasonable expectation that oligonucleotides complementary to the coding region of an mRNA could arrest translation of a specific protein.</u>

The examiner apparently believes that the cell-free systems of Hastie and Patterson provide one of skill with a reasonable expectation that *in vivo* arrest of protein expression was possible with oligonucleotides. This is not true. Both Hastie and Patterson used cDNA of lengths that exceed 500 bases. These DNAs required harsh denaturing conditions to effect binding to mRNA. The *in vivo* activity of oligonucleotides which are short DNA species, preferably between 13 to 23 bases, cannot be predicted by the *in vitro* behavior of cDNA. Furthermore the cDNA used by both Hastie and Patterson could not be used for *in vivo* arrest of protein expression. They are too long to cross a cell membrane.

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[1] Dr. Miller states in Blake *et al.* at page 6137, column 2, "The results of this study show that sequence-specific oligodeoxynucleotides can be used to arrest translation of specific mRNAs in a selective manner in cell-free systems."

[2] At page 1995, Miller states: "neutral oligonucleotide analogues with longer chain lengths and a greater resistance to hydrolysis would be expected to achieve a greater specificity and effectiveness in regulating the function of target nucleic acids inside the cell over a long time period."

In addition to the impropriety of comparing cDNA to oligonucleotides, there are a variety of additional objective reasons why there was no reasonable expectation in 1981 that oligonucleotides complementary to the coding region of mRNA could arrest translation of specific proteins *in vivo.* The osmotic potentials, the salt and pH conditions, the microenvironments within the various compartments of the cells, the ability of oligonucleotides to bind divalent cations and affect the electropotential and homeostasis of a cell, the secondary structure of the mRNA *in vivo*, the physical pressure and gel-like consistency of the cytosol, are all factors which precluded the ability of one of skill to predict with a reasonable certainty that complementary oligonucleotides could bind with sufficient strength, if at all, to the coding regions of mRNA to selectively arrest translation. As Dr. W. Salser succinctly stated in his 1978 review article, "The problem is then to assess which parts of the [mRNA] structure in Figure 3 may actually exist in the complex milieu of the cell."

Furthermore, even if one were to ignore the conventional wisdom that the secondary structure of mRNA rendered the coding region inaccessible to complementary oligonucleotides, there were a number of unknown parameters which alone or in combination render the question of successful regulation of expression by the claimed methods unpredictable. More specifically, there were a number of valid reasons why the oligonucleotides expected to arrest translation might not physically reach the mRNA inside a living cell. The mRNA of eukaryotes is produced in membrane bound nuclei. The art demonstrated that oligonucleotides would be taken up by cells, but not whether the oligonucleotides would be allowed access to the internal regions of the nuclei. Befort *et al.*, at page 184, states that exogenous oligonucleotides used as antiviral agents enter cells but are primarily bound to the microsomal fractions and do not significantly enter the host cell nuclei. It was known that messenger ribonucleic acid proteins bound to mRNA. According to Pain and Clemens, in *Comprehensive Biochemistry*, Vol 19B, Part 1 at pages 14-15 (1980), the function of these proteins was unknown. These proteins might have played a role in the storage and delivery of the mRNA to the correct ribosomes (membrane bound or not) and might have interfered with the access of the mRNA complementary oligonucleotides. As the authors go on to explain, only

10% of the total mRNA produced by the cells is actually used by the ribosomes to produce protein. That unknown mechanism, perhaps controlled by messenger ribonucleoprotein, as well as other unpredictable aspects of mRNA transport, could very well have rendered the mRNA unaccessible to complementary oligonucleotides.

In addition, there were the unknown effects of spermidine and spermine. These common polyamines tightly bind to the phosphate backbone of mRNA *in vivo* and play a role in the structure and function of mRNA at the ribosomes during protein synthesis. The impact of spermidine on the ability of oligonucleotides to bind *in vivo* to mRNA, either because the polyamines binding to the oligonucleotides might prevent hybridization to mRNA, or because spermidine bound to mRNA might block oligonucleotide binding *in vivo*, was simply unknown. Thus, the effect of spermidine and spermine was yet another unknown factor that would lead one of skill away from a reasonable expectation that one could effect the *in vivo* arrest of translation by oligonucleotides complementary to the coding region of specific mRNA.

In conclusion, it is clear that one of skill would not have expected with any reasonable degree of certainty in 1981 that an oligonucleotide specific for the coding region of an mRNA could arrest translation. In summary, the following seven objective reasons were identified above: (a) that the intact mRNA might not be physically accessible to complementary oligonucleotides; (b) that secondary structure of mRNA might block complementary oligonucleotide binding; (c) that short oligonucleotides might not have sufficient binding strength to block a ribosome designed to untangling internal duplexes in mRNA; (d) that the use of unsuitably long complementary oligonucleotides would have their own secondary structure that would interfere with hybridization of mRNA; (e) that polyamines and transport proteins might have rendered the coding region of mRNA inaccessible; (f) that the ability of oligonucleotides to bind cations might have had a toxic effect on target cells; and (g) that the majority of mRNA are not actually translated by cells but rapidly turned over.

This Declarant has nothing further to say.

Dated: 23 August 1994

Jerry L Ruth

Jerry L. Ruth, Ph.D.

attachments:
Exhibit 1 [Ruth C.V.]
Exhibit 2 [Schwartz C.V.]
Exhibit 3 [Ohtsuka *et al.* 1980]
Exhibit 4 [Ohtsuka *et al.* 1981]
Exhibit 5 [Gumport *et al.* 1980]
Exhibit 6 [Gumport and Uhlenbeck 1981]
Exhibit 7 [Miller *et al.* 1977]
Exhibit 8 [Miller *et al.* 1977]
Exhibit 9 [Efstratiadis *et al.* 1977]

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Box AF, Washington, D.C. 20231, on April 14, 1995

By [signature]

PATENT

Attorney Docket No. 16243-1-5

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Richard H. Tullis

Serial No.: 08/078,768

Filed: June 16, 1993

For: OLIGONUCLEOTIDE THERAPEUTIC AGENT AND METHODS OF MAKING SAME

Examiner: J. Martinell

DECLARATION OF JERRY L. RUTH

Box AF
Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Dr. Jerry L. Ruth, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true and statements made on information or belief are believed to be true. The Exhibits attached hereto are numbered 1-9. They are each incorporated herein by reference.

2. I have previously submitted a declaration in support of this patent application. My scientific credentials are presented therein.

3. As I understand the Examiner's remaining rejection, he believes that the pending claims should be restricted to the phosphotriester-modified nucleic acids that are stated as a preferred embodiment representing a class of stabilized nucleic acids for *in vivo* applications. The Examiner reasons that the pending claims inclusive of *in vivo* uses are too broad. More specifically, the Examiner states that no other suitable nucleic acid analogs were available as of the filing date in October of 1981, that unmodified nucleic acids would not be adequately stable to have biological activity under *in vivo* conditions, and that the means for administering the antisense molecules would require undue experimentation.

The purpose of this declaration is to address each of the above issues. More specifically, I will explain: (1) that as of the priority filing date, those of skill were aware of other analogs of nucleic acid that were modified to enhance stability against nuclease activity and which were suitable for *in vivo* use and that those of skill would have recognized from the Applicant's disclosure that these known analogs of nucleic acids were useful in the invention; (2) that the stability of natural (unmodified) nucleic acid under *in vivo* conditions is sufficient to permit the observation of its biological activity, *i.e.*, to inhibit expression of specific protein; and, (3) given the level of skill in the art, there is nothing but routine experimentation involved in the *in vivo* use of the claimed method.

A. PRIOR TO THE FILING DATE OF THE PARENT APPLICATION ON OCTOBER 23, 1981, A NUMBER OF STABILIZED NUCLEIC ACID ANALOGUES WERE AVAILABLE FOR USE IN THIS INVENTION.

1. The alkylphosphotriester DNA analogs described in the application as an example of a stabilized oligonucleotide were described in the literature in 1974 by Miller *et al.* (A1). These analogs have a phosphate bearing four oxygens, three of which are substituted with carbon-based substituents. The following discussion presents other references that describe chemically modified nucleic acids that were available prior to October of 1981 and were used intracellularly. Collectively, they present uncontestable evidence that a variety of stabilized nucleic acids were

known and available for use in the claimed invention as of the original filing date.

A second chemically modified nucleic acid was the methylated ribonucleic acids described by Befort *et al.*(1974). Befort is already of record as reference A27. In Befort, the authors reported uptake of their stabilized RNA into fibroblasts and the subsequent inhibition of viral multiplication. The stabilized nucleic acid was a methylated RNA that complemented a portion of the viral genome.

In Tennant *et al.* (1974), the authors describe the *in vivo* effects of an alkylated homopolymer of ribonucleic acid on virally induced oncogenesis. Tennant is already of record as reference A47.

In Kunkel *et al.* (Exhibit 1), *P.N.A.S. USA* 78(11):6734 (1981). The authors describe work conducted and published before 1981 using thio-substituted deoxynucleosides. On column 2 of page 6734, the authors describe that their analogs were previously reported as incorporated into oligonucleotides using DNA polymerase and nuclease resistant.

Finally, Miller *et al.* reported on the *in vivo* effects of a DNA analog in March 1981. This reference is already of record as A2. Attached to this declaration as Exhibit 2 is a true copy of the Medline abstract entry for this reference. The entry clearly identifies its publication date as March 1981, seven months before applicant's filing date. The analog described by Miller in 1981 was an alkyl phosphonate which differs from the phosphotriester of their earlier work by the direct attachment of the alkyl substituent to the phosphate. The Examiner is asked to review page 1879, second column, where a discussion of the intracellular half-lives of the phosphonates and the triesters are compared.

Thus, it is clear that as of the priority filing date of the present application, those of skill would have understood the applicant's reference to stabilized nucleic acid to have included more than the phosphotriester compounds that were specifically identified. Moreover, and because the use of stabilized nucleic acids was a mere example in a universe where both stabilized and natural oligonucleotides would function to downregulate expression of protein under *in vivo* conditions, it was, in my opinion, unnecessary to identify for those of skill all the stabilized nucleic acids that were available for use in the invention as of

October of 1981.

2. It is my understanding that the Examiner was concerned that the specification as filed would not have suggested that the nucleotide analogs described in the above references were useful in the invention. There are several objective reasons, why those of skill in 1981 would have understood that the text of the specification, ~~i.e., page 4, lines 8-13 and claims 29-33 of the~~ original specification was referring to the above identified body of knowledge.

The above referenced text of the applicant's disclosure states:

> The preferred oligonucleotide ... , for increased stability, may be transformed into a more stable form, such as a phosphotriester form, to inhibit degradation during use.

Original claims 29 and 32 recite stable forms of olignucleotides that inhibit degradation by organisms and claims 30 and 33 recite phosphotriester forms. The applicant clearly is teaching that stable, nuclease resistant forms of nucleic acid which can duplex to target nucleic acid are preferred forms of the oligonucleotides useful in the invention. Even presuming that those of skill were unaware of the above body of knowledge, to the best of my knowledge, there was no other body of knowledge to which the applicant could have been referring.

Having explained that there was no other body of knowledge that might have been confused with the above reference body of knowledge, it is simply a matter of establishing that one of skill would had the skill to locate the above references. The above references are representative of a significant body of work involving stabilized nucleic acids for understanding enzyme mechanics, transcription studies, for evaluting cellular uptake of nucleic acid and for medical uses. For the Examiner to maintain that those of skill would not have known of the above references or not have been able to find the above references is contrary to the way scientists work and contrary to my understanding of how the Patent Office establishes obviousness. The phosphotriester reference in the original application would have lead one of skill directly to Dr. Paul Miller's work and thus to other analogs. Dr. Miller's published work involved both phosphotriester and phosphonates analogs. Anyone familiar with Dr. Miller's work would have known

of analogous work by Dr. Fritz Eckstein using thio-substituted nucleic acid. Even undergraduates were being taught in 1981 that methylation was a key modification to nucleic acid for the purpose of increasing its half-life. In addition, the Examiner is asked to review the work of Dr. Summerton in 1978. This reference is already of record (A33). At page 89, Dr. Summerton summarized the art of modified nucleic acids for inhibiting viral replication and specifically addressed degradation problems. Among the modified nucleic acids taught by Summerton as useful as *in vivo* antiviral agents were the methylated oligonucleotides, thio-substituted nucleic acids as well as the modified olignucleotides of Miller.

Finally, as one of skill in 1981, I can state unequivocaly that I would have fully understood what nucleic acids analogs were being referred to by the applicant in his original application. For the above reasons, I am of the opinion that the Examiner's concern regarding whether one of skill would have understood the applicant's teaching to be inadequate to identify other modified nucleic acids is groundless.

B. UNMODIFIED RNA AND DNA HAVE A HALF-LIFE *IN VIVO* THAT IS SUFFICIENT TO PERMIT ITS UPTAKE INTO CELLS.

The Examiner questions whether unmodified nucleic acid will actually survive under *in vivo* conditions for a sufficient length of time to actually be taken up by the cells. There are numerous studies that should convince the Examiner that his concerns are without foundation. Unmodified nucleic acid does survive in the body for a fairly long period. While its susceptibility to degradation make more stable forms of nucleic acid a preferred embodiment, susceptibility to degradation does not render unmodified nucleic acid useless. It is merely a matter of dosage with unmodified nucleic acid requiring higher amounts and/or longer administration to see the equivalent effects of stabilized nucleic acid.

While most articles report on the use of DNA, unmodified RNA will also survive under *in vivo* conditions. In Michelson *et al.* (1985) "Poly(A)·Poly(U) as Adjuvant in Cancer Treatment Distribution and Pharmacokinetics in Rabbits

(42082)," *Proc. Soc. Exp. Biol. & Med.* 179:180-186 (Exhibit 3). In Michelson *et al.*, the authors describe the half-life of synthetic polyribonucleotides as measured in days. Its uptake into cells was also reported on page 184, 1st Col. A second report of long term survival of RNA appeared in Wolff *et al.* (1990) "Direct Gene Transfer into Mouse Muscle in Vivo," *Science*, 247:1465-1468 (Exhibit 4). In Michelson, purified RNA and DNA were simply injected into the muscle of mice and their respective gene products measured. The authors clearly state in their abstract that, "protein expression was detected in all cases and **no special delivery system was required....**"

Reports involving the use of purified DNA are more numerous than of RNA. Illustrative reports of DNA expression of plasmids directly injected into animals are provided in Lin *et al.* (1990) "Expression of Recombinant Genes in Myocardium In Vivo After Direct Injection of DNA," *Circulation*, 82:2217-2221 and Wolff *et al.* (1992) "Long-Term Persistence of Plasmid DNA and Foreign Gene Expression in Mouse Muscle," *Human Mol. Genet.* 1(6):363-369 which are Exhibits 5 and 6, respectively. The DNA may be linear or circular (see Exhibit 4 at page 368, 2nd Col.)

C. UNMODIFIED ANTISENSE OLIGONUCLEOTIDES HAVE BEEN DEMONSTRATED TO BE USEFUL IN A VARIETY OF DIFFERENT ORGANS.

Naked, natural phosphodiester, antisense oligodeoxynucleotides have been reported as sufficiently stable to downregulate gene expression when directly injected into an animal. For example, Phillip *et al.* (1994) "Antisense Inhibition of Hypertension: A new strategy for Renin-Angiotensin Candidate Genes," *Kidney Intern.*, 46:1554-1556 (Exhibit 7) reports on the direct injection of an antisense DNA (unmodified) for reducing hypertension in mice. The DNA was merely injected into the mouse carotid artery using a saline solution.

Others have reported that antisense DNA will work when directly injected into the brain. For example, in Akabayashi *et al.* (1994) *Mol. Brain Res. 21:55-61*, the authors dissolved the antisense DNA in saline and simply injected it into the

brain to inhibit production of a neuropeptide (Exhibit 8). At page 56, 1st Col., the authors state that theirs is the third such report.

As stated above, the use of stabilized DNA was merely a preferred embodiment. The use of unmodified DNA was less preferred, but similar results could be achieved by merely using more DNA or RNA to accommodate instability. Given the level of skill of those practicing molecular biology, this is an intuitively apparent solution to an obvious problem. The use of high levels of DNA is described in Exhibit 9, Hijya *et al.* (1994) *Proc. Natl. Acad. Sci., U.S.A.* 91:4499-4503. Hijya *et al.* report on the use of an unmodified phosphodiester oligonucleotide for controlling the expression of a gene which is involved in skin cancer. The authors applied the antisense oligonucleotide via a subcutaneous route and used constant-infusion pumps to ensure that the oligonucleotide was adequately administered.

D. THERE IS NO UNDUE EXPERIMENTATION INVOLVED IN THE ADMINISTRATION OF ANTISENSE OLIGONUCLEOTIDES.

The level of skill of those in the art of antisense technology is quite high. Most of the artisans are like myself and hold doctorates in a relevant biological science. To achieve a measurable downregulation of protein expression, one need only contact the target cells with an adequate amount of antisense oligonucleotides. The infusion techniques are conventional and were fully known in 1981. The technique is merely the injection of a saline solution containing the antisense oligonucleotides into the appropriate organ. There is simply no basis to conclude that such a experimental step was anything but routine and intuitively apparent to those of skill.

In summary, the relative stability of unmodified antisense oligonucleotides compared to stabilized oligonucleotides does not render the *in vivo* use of unmodified DNA or RNA without utility for the purpose of downregulating protein expression. The attached Exhibits 3-9 clearly document to one of skill that the claimed methods are operable under *in vivo* conditions. Furthermore, there is

nothing beyond routine experimentation required to administer the antisense oligonucleotides under *in vivo* conditions and detect a downregulation in the expression of a specific protein.

The declarant has nothing further to state.

Dated: 7 April 1995 By: Jerry L Ruth
Dr. Jerry L. Ruth

Attachments: Exhibits 1-9

I hereby certify that . correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231, on Aug. 19 1994

By Jo Ann Horvald Dallara

PATENT

Attorney Docket No. 16243-1-5

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Richard H. Tullis	Examiner: J. Martinell
Serial No.: 08/078,768	Art Unit: 1805
Filed: June 16, 1993	DECLARATION PURSUANT TO 37 C.F.R. § 1.132
For: OLIGONUCLEOTIDE THERAPEUTIC AGENT AND METHODS OF MAKING SAME	

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

I, Dr. Dennis E. Schwartz, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true and statements made on information or belief are believed to be true. The Exhibits (1-9) attached hereto are incorporated herein by reference.

2. I received a Ph.D. in chemistry and biological science from the Purdue University in 1972. I spent 10 years at Harvard University as a Postdoctoral fellow in the laboratories of Drs. Walter Gilbert (Nobel prize laureate) and Paul Zamecnik (member of the National Academy of Science). My research specialty involves nucleic acid chemistry and biology.

A copy of my curriculum vitae is attached as Exhibit 2.

3. I have been a scientific founder of two companies, MicroProbe Corporation and Origen. I am presently employed by Origen as a founder and am responsible for development of novel therapeutic compounds for human diseases.

4. I have read and am familiar with the contents of the application and related papers. I understand that the Examiner has made two rejections. The first rejection is for failure to fully teach how to make and use the invention as claimed, and the second is that the invention is obvious over a combination of four references. This declaration will address both rejections. I will provide objective evidence of the state of the art of arresting specific protein expression by oligonucleotide hybridization in 1981. The evidence will be provided by interpretation of references relating to this art and by my personal perspective from having been involved in the field of oligonucleotide chemistry and biology in 1981.

5. **ENABLEMENT**

A. <u>Analogs of Nucleic Acid</u>

It is my understanding that the Examiner has concerns that the invention should be limited to phosphotriester-modified nucleic acid and that claims reading on natural nucleic acid and other analogs are not enabled. It is my opinion that once Dr. Tullis identified functional sizes and the mRNA coding region as a target, the invention was fully disclosed to one of skill. The utility of natural nucleic acid and various analogs to be internalized by cells and inhibit cell function was known. Evidence of these facts can be found in the prior art. For example, Miller (1977) describes neutral, nonionic nucleic acids for nonspecific inhibition of protein synthesis. Before (1974) used methylated ribonucleic acid to inhibit viral replication. Zamecnik and Stephenson (1978) used a natural phosphodiester DNA tridecamer to inhibit viral replication *in vivo*. The DNA was analyzed for purity by me. Finally, Summerton (1979) described a number of early reports using various nucleic acid analogs to inhibit viral infections (see page 89).

B. <u>Making Ribonucleic Acid</u>

The Examiner further argues that there is no teaching of how to make or use ribonucleotides. The methodology for making synthetic RNA and its analogs was available in 1981. Evidence of this fact can be found in the Miller reference (1977). Therein the authors used an analog of ribonucleic acid to non-specifically inhibit protein synthesis.

Moreover, methods for chemical synthesis of oligoribonucleotides were well established by 1981 as is illustrated by reference to the work of Ohtsuka and his colleagues. In the mid and late 1970s, Ohtsuka's group reported synthesis of numerous oligoribonucleotides corresponding to the sequence of an *E. coli* tRNA. Some of this work is described in Nuc. Acids. Res. Symp. Series (NARS) No. 7, pp. 335-343 (1980), which is attached as Exhibit 3, and the cites therein. In 1980 Ohtsuka reported synthesis of oligonucleotides corresponding to the total sequence of *Escherichia coli* $tRNA_f^{met}$; these oligos were joined using RNA ligase to create an entirely synthetic tRNA (*Id.*). The synthesis of *E. coli* tRNA is also discussed in a subsequent paper that appeared prior to the filing date of the application [Proc. Nat. Acad. Sci. 78(9) 5493 (1981); attached as Exhibit 4]. Clearly, by 1981 methods for chemical synthesis had been available for several years.

Enzymatic methods for synthesis of oligoribonucleotides complement chemical methods and include use of polynucleotide phosphorylase and T4 RNA ligase. RNA ligase in particular has been useful in synthesis of oligoribonucleotides and in 1980 Gumport *et al.*, in a paper on T4 RNA ligase, observed that "...the enzyme is now widely used to synthesize defined sequences of RNA." [NARS No. 7 (1980) pp. 167-171 at 167; attached as Exhibit 5]. In a 1981 review attached as Exhibit 6, ("T4 RNA Ligase as a Nucleic Acid Synthesis and Modification Reagent" in *Gene Amplification and Analysis*, Vol. 2, Chirikjian and Papas, eds. Elsevier (New York) 1981, pp. 314-345 at pages 335-339), Gumport and Uhlenbeck describe work by several groups engaged in oligoribonucleotide synthesis, including Ohtsuka [described above], Neilson and colleagues [using a combination of organic and enzymatic methods to prepare several decanucleotides], Krug and colleagues [preparation of a 21-nucleotide RNA], and others [see citations at 337, first full paragraph]. In my opinion, the attached exhibits clearly demonstrate that methods for synthesis of oligoribonucleotides were well known in 1981.

C. Cell Uptake of Nucleic Acid

Finally the Examiner raised the issue of cell uptake of nucleic acid. He comments that there are no data and methods for actually "getting short DNAs or RNAs into cells." Living cells have a natural capacity for internalizing short nucleic acids. There is nothing to teach. The cells internalize these compounds without special culture conditions. There is no need to render the cells porous. The literature relied upon by the Examiner to support his obviousness rejection teaches this fundamental fact. For example, the Miller reference involves the effect of a trinucleotide analog on mammalian cells and the Summerton reference discloses at pages 93-94 the routine uptake by animal cells of both RNA and DNA. Finally there is the paper by Zamecnik and Stephenson (1978) which describes the internalization of viral infected cells by a DNA of 13 nucleotides.

6. **OBVIOUSNESS**

It is my understanding that the Examiner believes that in 1981 a person of skill reading Itakura *et al.*, Paterson *et al.* or Hastie *et al.* and Summerton or Miller *et al.* would have had a motivation and a reasonable expectation that targeting the coding region of a specific mRNA with a oligonucleotide complementary to the coding region would have arrested protein translation of that mRNA. There are a number of objective reasons why this is not an accurate statement of the state of the art in 1981.

A. The secondary structure of the mRNA made it an unlikely target for control of expression by complementary oligonucleotides.

The claimed invention went against the conventional wisdom of the time. The conventional wisdom in 1981 was that the secondary structure of mRNA was extensive that there was no reasonable likelihood that a oligonucleotide complementary to a coding sequence would have sufficient access to arrest translation inside a living cell. In addition, those of skill understood that the natural mechanism of peptide elongation by ribosomes involved the destabilization of the extensive secondary structure of the mRNA. For these reasons, the idea of hybridizing a complementary oligonucleotide to a coding region of mRNA to arrest translation was contrary to conventional wisdom. The oligonucleotide would have

to overcome two significant hurdles. First it had to bind to the coding region of the mRNA, which was viewed as a Gordian knot of secondary structure. And even if the complementary oligonucleotide could find and anneal to its complementary subsequence, the ribosomes were viewed as able to bind to a specific non-coding site and then read mRNA coding regions constrained by extensive secondary structure. Thus it was not likely that the hybridization of a complementary oligonucleotide would arrest translation. The ribosome would simply "toss" the duplexing structure aside.

Although there is little express language in the prior-art articulating the above concerns, the literature indirectly compels one to conclude that secondary structure and ribosomal helix destabilization were concepts teaching away from the invention. References published after the priority date of the subject application do expressly identify the state of the art at the time of the invention.

The extensive secondary structure of mRNA was well understood in 1981. In the book, The Ribonucleic Acids, 1977, Eds. Stewart and Latham, Springer Verlag, Chpt. 4, "Messenger RNA" by J.M. Adams, the extensive secondary structure in phage mRNA and eukaryotic mRNA is described. Those of skill recognized the importance of mRNA secondary structure and particularly were aware of the need of low mRNA secondary structure in the region where ribosomes initially bind to mRNA. W. Salser, in his chapter Globin mRNA Sequences: Analysis of Base Pairing and Evolutionary Implications, in *Chromatin* Vol XLII (1978) pages 985-1001 at page 992, provided a graphic illustration of the extensive amount of secondary structure in the typical mRNA. In Dr. Salser's illustration, the sequences involved in ribosome initiation and interaction are dramatically free of secondary structure. Dr. Adams, on page 103 in his section (e) entitled "Influence of secondary and tertiary structure on initiation," also discusses the importance of secondary structure on initiation of translation. In chapter 7, by Wettenhall and Clarke-Walker of *The Ribonucleic Acids*, the authors on page 258 in a section entitled "Messenger RNA structure" expressly state, "[t]he role of mRNA structure in initiation reactions is receiving considerable

attention." These authors also discuss the role of secondary structure of mRNA on translation efficiency.

The concern over availability of the coding region to bind oligonucleotides is made more apparent when one looks at the secondary references relied upon by the Examiner. These references are Paterson *et al.* and Hastie *et al.* describing cell-free, *in vitro* experiments in which denaturing conditions to relax the secondary structure of their mRNA are applied prior to hybridizing nucleic acids. For example, Patterson used 100°C for 30 seconds and Hastie used temperatures between 45°C and 65°C. For the Examiner to believe that one of skill would have understood that complementary oligonucleotides were able to bind to the coding regions of mRNA under *in vivo* conditions when both Hastie and Paterson used denaturing conditions is logically inconsistent and scientifically incorrect.

In 1980, I was personally working on a project that had similar aspects to Dr. Tullis' work. I was attempting to sequence the RNA genome of Rous Sarcoma Virus. I used a procedure which relied on *in vitro* hybridization of short oligonucleotides to the RSV-RNA, followed by 3' extension of the primers using reverse transcriptase. The resulting cDNA was then sequenced. Like my colleagues seeking to bind DNA to mRNA, the secondary structure of the RSV-RNA was of paramount concern to me. I took a number of deliberate steps to minimize the effect of secondary structure which might block primer attachment and prevent me from obtaining a complete set of cDNA species to sequence. Those steps included heating the viral RNA to 95° in low salt (to remove secondary structure), rapidly chilling the RNA on ice in a low salt buffer (to freeze the RNA in an open conformation), followed by the addition of reverse transcriptase and DNA primers as rapidly as possible (less than one minute), and running the reverse transcription at the highest possible temperature to maintain an open conformation. The work was published in *Cell*, 32:853-869 (1983), Exhibit 7. I declare that the concerns of secondary structure were known to me in 1981, are evidenced by my work published in 1983, and were of paramount concern to those of skill in the relevant art of the subject invention.

A number of other references taught that the targeting of a coding regions would not be a preferred target for a oligonucleotide agent expected to control expression. Pluskal *et al. Biochem. Soc. Trans.* 7:1091-1093 (1979), wrote that their work with a heterogenous mixture of low molecular weight oligonucleotides non-specifically inhibited translation, but that the mode of action was not affected by preincubation with the mRNA. One of skill is left to conclude that the translation inhibition observed by Pluskal did not occur via complementary binding interactions between the oligonucleotides and the mRNA.

Continuing in temporal order, the Ts'o patent further suggests away from the Applicant's invention. In the Ts'o patent, the claimed oligonucleotide analogues are described as inhibiting expression of a preselected sequence, either a cellular nucleic acid or a viral nucleic acid. Ts'o uses trimer analogues and at column 25, lines 5-10, they state that their analogues inhibited poly(U) messages but not globin messages. In column 26, lines 5-34, Ts'o explains that they saw no inhibition *in vivo* of either bacteria or hamster cells.

Ts'o's results are consistent with later published reports. These later reports actually explain what was intuitively apparent to those of ordinary skill at the time Dr. Tullis filed his application in 1981. There was no objective reason or basis to conclude that the coding region of mRNA was an effective target for *in vivo* control of expression by trinucleotides because there was no evidence that the trimers used by Ts'o inhibited elongation when bound to mRNA either *in vitro* or *in vivo*. Either the coding regions of mRNA were physically unavailable to complementary oligonucleotides or even if binding to the coding regions, the oligonucleotides were unable to arrest the elongation process of the ribosomes. As explained above, the latter concept was particularly compelling because to elongate, ribosomes have to untwist (denature) the secondary structure of mRNA.

Perhaps the most express and compelling articulation of the true state of the art from 1977 until after the effective filing date of the subject application is found in Dr. Miller's own work published in two parallel reports in 1985. In Blake, Murakami and Miller, *Biochemistry 24*, 6132-6138 and *24*, 6139-6145, the authors discuss at length their concerns over secondary structure on the

availability of subsequences of mRNA to complementary oligonucleotides. In the first of the two companion articles (A22), Dr. Miller looked at 8-12 mer oligonucleotides and concluded, on page 6135, that in the cell free rabbit reticulocyte protein expression system, 8-mer oligonucleotides do not arrest protein elongation, but that if bound to the initiation codon region the oligonucleotides will arrest translation. On the second column of page 6136, Dr. Miller in summarizing the state of the art expressly states:

> The above results are in agreement with the recent findings of Liebhaber *et al.* (1984). They found that cDNAs to human globin mRNA which cover the initiation codon or extend into the 5'-noncoding region are able to completely inhibit translation in a rabbit reticulocyte lysate. In contrast cDNAs which cover only the coding region exclusive of the initiation site are not effective at blocking translation. These authors postulate that a helix-destabilizing activity associated with the reticulocyte ribosomes is able to disrupt secondary structure during the elongation step but not the initiation step of translation. **Thus, cDNAs or oligonucleotides bound to the coding region of mRNA would be expected to be unable to prevent translation in the reticulocyte system.** [Emphasis added]

Dr. Miller is summarizing two points to explain why oligonucleotides binding to the initiation codons will inhibit translation but those binding to coding regions will not inhibit translation. He first explains that the ribosomes will simply remove the oligonucleotides bound to the translated region (page 6136, column 2), and second, he explains that the secondary structure of mRNA precludes the binding of the oligonucleotides to the translated region (page 6137). He implies this is true even for cDNAs which may be hundreds of bases long.

In the second article, Dr. Miller is again reporting on the ability of oligonucleotides to arrest *in vitro* translation. In this report the oligonucleotides are between 6 and 11 bases long. The authors are reporting some degree of success with the binding of oligonucleotides to mRNA coding regions. Although the success of the method is being reported, the authors are clearly articulating the concerns over secondary structure which was the conventional wisdom in 1981. For example at page 6144, the authors state:

> In addition to the effect of mRNA secondary structure, the region of the mRNA to which the oligonucleotide methylphosphonates binds influences its effectiveness as an inhibitor of mRNA translation. The results in Table II suggest that oligomers complementary to the 5' end and initiation codon regions are somewhat better inhibitors than the oligomers which bind to the coding regions. **Thus it appears that the initiation step of translation is more sensitive to oligomer mRNA binding than the elongation step of translation.** [Emphasis added]

Finally, as late as 1986, the literature was still suggesting that short oligonucleotides will only arrest translation if bound to the initiation sites. In particular, Haeuptle *et al.,* using a cell free translation system, clearly states that size of the oligonucleotides bound to coding regions is a critical key to ensuring arrest of translation. Just as is taught by the subject application with its effective filing date of 1981, Haeuptle reports in 1986 that the oligonucleotides must be 10 bases or greater in length before substantial inhibition of translation is demonstrated. The Examiner should also note that the authors, in deference to secondary structure, are using 55°C for 5 minutes to relax the mRNA sufficiently to allow the oligonucleotides to bind to their target regions.

In conclusion, I state that the conventional wisdom in the art, at the time of the invention, taught away from Dr. Tullis' invention. The literature cited above is offered as evidence of this wisdom. This literature clearly teaches that the secondary structure of mRNA, especially in the coding region, was thought by those of skill in 1981 to be extensive and that this secondary structure would preclude binding of a complementary oligonucleotide to the coding region mRNA for the purpose of arresting translation. Also, there was reason to believe that the helix destabilization of ribosomes during elongation would have easily displaced any oligonucleotide duplexed on the coding region. Therefore, one of skill would not have considered the claimed invention to have a reasonable expectation of success in 1981 when Dr. Tullis originally filed the subject patent application.

B. <u>The Examiner's interpretation of the text in Miller (1977) goes well beyond the understanding one of skill would have reading the same text in 1981.</u>

It is my further understanding that the Examiner has stated that Miller (1977) expressly states that oligonucleotides complementary to the coding region

of mRNA might inhibit cellular protein synthesis *in vivo*. The two statements have been cited by the Examiner. The abstract states:

> Our biochemical studies suggest that inhibition of cellular protein synthesis might be expected if $G^mp(Et)G^mp(Et)U$, $G^mp(Et)G^mpU$, and G^mpG^mpU, which have been taken up by or formed within the cell, physically bind to tRNA and mRNA and inhibit the function of these nucleic acids.

The last paragraph states:

> The triester $G^mp(Et)G^mp(Et)U$ is complementary to target nucleic acids involved in cellular protein synthesis and has a transitory effect on the cells which is then relieved by its degradation. Such a transitory effect of inhibition may provide a convenient and useful way of imposing a temporary interruption of cellular functions. On the other hand, neutral oligonucleotide analogues with longer chain lengths and a greater resistance to hydrolysis would be expected to achieve a greater specificity and effectiveness in regulating the function of target nucleic acids inside the cell over a long period of time. The observations of the present study lay the groundwork for continued investigation into the use of neutral oligonucleotide analogues as proves and regulators of nucleic acid function within living cells.

There are multiple reasons why one of skill in 1981 would not have interpreted the cited text in the way the Examiner suggests. The text clearly does not state that oligonucleotide analogs could bind to the coding regions of mRNA. At best it is ambiguous when taken out of context both literally and historically. It is literally ambiguous because the authors' work is directed to general inhibition of all protein expression by using a trimer to bind to the amino acid accepting codon of a tRNA. There is no evidence of binding to target mRNA or of any inhibition of specific protein expression due to complementary binding of Miller's trimer to mRNA.

The phrase "greater specificity" is patently ambiguous. Statistically, any trimer sequence is represented every 64 nucleotides and therefore a trimer binds nonspecifically. Miller's use of "greater specificity" could refer to oligonucleotides that are specific (only) for the amino acid accepting codon of tRNA or that bind specifically (only) to the initiation region of a mRNA, or thirdly, as the Examiner would read the passage, using longer oligonucleotides that would bind with greater specificity to the coding region of an mRNA.

In historical context, the meaning of the above quoted text is clearly directed to either the tRNA or mRNA binding sites. These were only regions perceived by scientists in 1981 as being sufficiently accessible to complementary oligonucleotide binding under *in vivo* conditions. As explained above, the extensive secondary structure of mRNA was a primary reason why those of skill would have avoided the coding region of mRNA to arrest translation. For example, in Dr. Salser's review article of 1978, he includes a figure (Figure 3) depicting the proposed secondary structure of a mRNA. It is an extraordinary complex and twisted structure. Only a few regions are accessible to a complementary oligonucleotide and these are designated as points of initiation and tRNA interactions. These open regions which are generally found as single-strand hairpin loops.

Furthermore, the historical record indicates that by April of 1981, Dr. Mill understood the limitations of his 1977 report. In *Biochemistry*, 20:1874-1880, Exhibit 8, Dr. Miller *et al.* reported on the arrest of globin expression via blockage of tRNA aminoacylation by trimers and tetramers. On page 1879, and at Table VI, they report no effect on globin synthesis despite the fact that the oligonucleotides used by Miller (polyA) could bind to at least three sites in the coding region of mRNA encoding globin (codons 45, 85 and 118). See Exhibit 9.

Finally, in 1985, the last two Miller references summarize the historical evidence that taught away from targeting the coding regions of mRNA with oligonucleotide to arrest translation. First, in the introductions to both 1985 papers, there is no reference to the 1977 paper when discussing mRNA as a target for control of protein expression by complementary oligonucleotides. Secondly, as stated above on pages 7-8, the 1985 references specifically suggest; (1) that secondary structure will prevent binding of oligonucleotides to mRNA; and, (2) that helix destabilizing properties of ribosomes will remove oligonucleotide even it they were to have access to the coding region of an mRNA. These are two conclusive reasons why one of skill would not expect the arrest of protein translation by oligonucleotide binding to the two conclusive coding regions of mRNA.

In summary, by 1981, Miller's reference to "greater specificity" would not have been interpreted as a suggestion for controlling the expression of particular "target" proteins by binding to specific coding regions of mRNA, but as a suggestion to use longer oligonucleotides to bind **specifically** to the open regions of the tRNA or mRNA that bind to rRNA during the initiation step of protein synthesis. The open binding sites are longer than three bases and thus one would expect greater specificity for binding by using oligonucleotides greater than three bases. For these reasons, I state definitively that one of skill reading Miller in 1981 would have recognized that the proposed targets for binding oligonucleotides are these binding sites.

To argue that in 1981, one of ordinary skill would have predicted *in vivo* utility for an oligonucleotide binding to the coding portions of mRNA is to read Miller in a vacuum, and ignores the authors' data and the historical understanding of the accessibility of the coding region of mRNA due to secondary structure.

C. Although the Examiner reads the Miller reference as suggesting the use of an oligonucleotide binding to the coding region of an mRNA, Miller does not suggest this aspect of Dr. Tullis' invention.

The Miller reference is silent as to the target sequences on a mRNA to which its oligonucleotides might bind. Messenger RNAs have several functional domains. The coding region is only one domain. These various domains are described as "signals" by W. Salser in his chapter Globin mRNA Sequences: Analysis of Base Pairing and Evolutionary Implications, in *Chromatin* Vol XLII (1978) pages 985-1001. The signal sequences are described on page 988 as including "ribosome binding, mRNA processing, transport from the nucleus to the cytoplasm and so on".

Dr. Tullis' claims involve only the coding region and one reading Miller would not have been directed to this region. One of skill with knowledge of the secondary constraints of the coding region of mRNA and the mechanism by which ribosomes read mRNA would not have read Miller as suggesting the targeting of the mRNA coding region.

D. <u>Even if the prior art oligonucleotides of Miller were binding to the coding region of an mRNA, they could not have arrested translation.</u>

As further evidence that the Examiner's interpretation of Miller goes beyond any reasonable interpretation of the reference by one of skill in 1981, I would like to point out that the inhibition detected by Miller was in fact solely due to a non-specific interference of tRNA. We know this because Miller uses only trimer oligonucleotides and trimers were later shown to be useless as inhibitors of translation even if bound to a coding region of an mRNA.

The Examiner is directed to the 1986 reference of Haeuptle *et al.* Haeuptle describes the *in vitro* arrest of translation by oligodeoxyribonucleotides. The authors relaxed the secondary structure of mRNA encoding lysozyme using 55°C and hybridized oligonucleotides of varying lengths to a portion of the coding region. On page 1435 and in Figure 4 on page 1439, they present evidence of their inability to arrest translation using short oligonucleotides of 5 bases. The 5-mer species are simply too small to have sufficient binding strength to arrest the progress of a ribosome. The authors reported successful arrest of translation with their 10, 15 and 20 mer species.

E. <u>The Examiner has misinterpreted the use of "specificity" by Miller in 1977.</u>

The Examiner relies on the final paragraph of Miller, 1977 stating: "... oligonucleotide analogs with longer chain lengths and a greater resistance to hydrolysis would be expected to achieve a greater *specificity* and effectiveness in regulating the function of target nucleic acids...". The Examiner reads this sentence to include a suggestion to use oligonucleotides for longer binding to the coding regions of mRNA. Above I have explained that this phrase is and would have been understood by one of skill in 1981 to be directed to the non-coding regions of mRNA. I would like to focus this part of my declaration on evidence that the authors' reference to "specificity" was in a general context and did not refer to the coding regions of mRNA encoding specific proteins. More particularly, the authors were referring to oligonucleotides that bound more specifically to the amino acid accepting end of the tRNA and the initiation sites of mRNA.

Dr. Miller's later publication, Blake *et al.* (1985A) provides evidence of his intended meaning in using "specificity" in 1977. On page 6137, column 2 is the word "specific[1]" used in the same context that the word "specificity[2]" was used in 1977. Miller is again asserting that his work suggests *specific* control of expression by binding oligonucleotides to mRNA. But the context of the 1985 reference clearly implies that the arresting oligonucleotides are not directed to the coding regions of the mRNA. The Blake article states that the only available regions are the initiation points due to secondary structure. Thus the Examiner's interpretation of the text of Miller (1977) in an unlimited and broad context so as to include the coding regions of mRNA is to ignore the scientific realities of the contemporaneous literature.

F. <u>There are objective scientific reasons why one of skill would have not had a reasonable expectation that oligonucleotides complementary to the coding region of an mRNA could arrest translation of a specific protein.</u>

The examiner apparently believes that the cell-free systems of Hastie and Patterson provide one of skill with a reasonable expectation that *in vivo* arrest of protein expression was possible with oligonucleotides. This is not true. Both Hastie and Patterson used cDNAs of lengths that exceed 500 bases. These DNAs required harsh denaturing conditions to effect binding to mRNA. The *in vivo* activity of oligonucleotides which are short DNA species, preferably between 13 to 23 bases, cannot be predicted by the *in vitro* behavior of cDNA. Furthermore, the cDNA used by both Hastie and Patterson are too long to cross a cell membrane. They could not be used for *in vivo* control of translation.

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1 Dr. Miller states in Blake *et al.* at page 6137, column 2, "The results of this study show that sequence-specific oligodeoxynucleotides can be used to arrest translation of specific mRNAs in a selective manner in cell-free systems."

2 At page 1995, Miller states: "neutral oligonucleotide analogues with longer chain lengths and a greater resistance to hydrolysis would be expected to achieve a greater specificity and effectiveness in regulating the function of target nucleic acids inside the cell over a long time period."

In addition to the impropriety of comparing cDNA to oligonucleotides, there are a variety of additional objective reasons why there was no reasonable expectation in 1981 that oligonucleotides complementary to the coding region of mRNA could arrest translation of specific proteins *in vivo.* The osmotic potentials, the salt and pH conditions, the microenvironments within the various compartments of the cells, the ability of oligionucleotides to bind divalent cations and affect the electropotential and homeostasis of a cell, the secondary structure of the mRNA *in vivo*, the physical pressure and gel-like consistency of the cytosol, are all factors which precluded the ability of one of skill to predict with a reasonable certainty that complementary oligonucleotides could bind with sufficient strength, if at all, to the coding regions of mRNA to selectively arrest translation. As Dr. W. Salser succinctly stated in his 1978 review article, "The problem is then to assess which parts of the [mRNA] structure in Figure 3 may actually exist in the complex milieu of the cell."

Furthermore, even if one were to ignore the conventional wisdom that the secondary structure of mRNA rendered the coding region inaccessible to complementary oligonucleotides, there were a number of unknown parameters which alone or in combination render the question of successful regulation of expression by the claimed methods unpredictable. More specifically, there were a number of valid reasons why the oligonucleotides expected to arrest translation might not physically reach the mRNA inside a living cell. The mRNA of eukaryotes is produced in membrane bound nuclei. The art demonstrated that oligonucleotides would be taken up by cells, but not whether the oligonucleotides would be allowed access to the internal regions of the nuclei. Before *et al.*, at page 184, states that exogenous oligonucleotides used as antiviral agents enter cells but are primarily bound to the microsomal fractions and do not significantly enter the host cell nuclei. It was known that messenger ribonucleic acid proteins bound to mRNA. According to Pain and Clemens, in *Comprehensive Biochemistry*, Vol 19B, Part 1 at pages 14-15 (1980), the function of these proteins was unknown. These proteins might have played a role in the storage and delivery of the mRNA to the correct ribosomes (membrane bound or not) and might have interfered with the access of

the mRNA complementary oligonucleotides. As the authors go on to explain, only 10% of the total mRNA produced by the cells is actually used by the ribosomes to produce protein. That unknown mechanism, perhaps controlled by messenger ribonucleoprotein, as well as other unpredictable aspects of mRNA transport, could very well have rendered the mRNA unaccessible to complementary oligonucleotides.

In addition, there were the unknown effects of spermidine and spermine. These common polyamines tightly bind to the phosphate backbone of mRNA *in vivo* and play a role in structure and function of mRNA and ribosomes during protein synthesis. The impact of spermidine on the ability of oligonucleotides to bind *in vivo* to mRNA, either because the polyamines binding to the oligonucleotides might prevent hybridization to mRNA, or because spermidine bound to mRNA might block oligonucleotide binding *in vivo*, was simply unknown. Thus, the effect of spermidine and spermine was yet another unknown factor that would lead one of skill away from a reasonable expectation that one could effect the *in vivo* arrest of translation by oligonucleotides complementary to the coding region of specific mRNA.

In conclusion, it is clear that one of skill would not have expected, with any reasonable degree of certainty, in 1981 that an oligonucleotide specific for the coding region of an mRNA could arrest translation. In summary, the following seven objective reasons were identified above: (a) that the intact mRNA might not be physically accessible to complementary oligonucleotides; (b) that secondary structure of mRNA might block complementary oligonucleotide binding; (c) that short oligonucleotides might not have sufficient binding strength to block a ribosome designed to untangling internal duplexes in mRNA; (d) that the use of unsuitably long complementary oligonucleotides would have their own secondary structure that would interfere with hybridization of mRNA; (e) that polyamines and transport proteins might have rendered the coding region of mRNA inaccessible; (f) that the ability of oligonucleotides to bind cations might have had a toxic affect in target cells; and (g) that the majority of mRNA are not actually translated by cells but rapidly turned over--the flooding of a living cell with oligonucleotides at the

concentrations necessary to effectively bind to mRNA might have been toxic in a non-specific way.

This Declarant has nothing further to say.

Dated: August 17, 1994

Dennis E. Schwartz
Dennis E. Schwartz, Ph.D.

attachments:
Exhibit 1 [Ruth C.V.]
Exhibit 2 [Schwartz C.V.]
Exhibit 3 [Ohtsuka *et al.* 1980]
Exhibit 4 [Ohtsuka *et al.* 1981]
Exhibit 5 [Gumport *et al.* 1980]
Exhibit 6 [Gumport and Uhlenbeck 1981]
Exhibit 7 [Schwartz *et al.* 1983]
Exhibit 8 [Miller *et al.* 1981]
Exhibit 9 [Efstratiadis *et al.* 1977]

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I hereby certify that this c spondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Box AF, Washington, D.C. 20231, on April 14 1995

By [signature]

PATENT

Attorney Docket No. 16243-1-5

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Richard H. Tullis	Examiner: J. Martinell
Serial No.: 08/078,768	DECLARATION OF DENNIS E. SCHWARTZ
Filed: June 16, 1993	
For: OLIGONUCLEOTIDE THERAPEUTIC AGENT AND METHODS OF MAKING SAME	

Box AF
Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Dr. Dennis E. Schwartz, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true and statements made on information or belief are believed to be true. The Exhibits attached hereto are numbered 1-9. They are each incorporated herein by reference.

2. I have previously submitted a declaration in support of this patent application. My scientific credentials are presented therein.

3. As I understand the Examiner's remaining rejection, he believes that the pending claims should be restricted to the phosphotriester-modified nucleic acids that are stated as a preferred embodiment representing a class of stabilized nucleic acids for *in vivo* applications. The Examiner reasons that the pending claims inclusive of *in vivo* uses are too broad. More specifically, the Examiner states that no other suitable nucleic acid analogs were available as of the filing date in October of 1981, that unmodified nucleic acids would not be adequately stable to have biological activity under *in vivo* conditions, and that the means for administering the antisense molecules would require undue experimentation.

The purpose of this declaration is to address each of the above issues. More specifically, I will explain: (1) that as of the priority filing date, those of skill were aware of other analogs of nucleic acid that were modified to enhance stability against nuclease activity and which were suitable for *in vivo* use and that those of skill would have recognized from the Applicant's disclosure that these known analogs of nucleic acids were useful in the invention; (2) that the stability of natural (unmodified) nucleic acid under *in vivo* conditions is sufficient to permit the observation of its biological activity, *i.e.*, to inhibit expression of specific proteins; and, (3) that given the level of skill in the art, there is nothing but routine experimentation involved in the *in vivo* use of the claimed method.

A. PRIOR TO THE FILING DATE OF THE PARENT APPLICATION ON OCTOBER 23, 1981, A NUMBER OF STABILIZED NUCLEIC ACID ANALOGUES WERE AVAILABLE FOR USE IN THIS INVENTION AND KNOWN THOSE OF SKILL.

1. The alkylphosphotriester DNA analogs described in the application as an example of a stabilized oligonucleotide were described in the literature in 1974 by [redacted]. These analogs have a phosphate bearing four oxygens, three of which are substituted with carbon-based substituents. The following discussion presents other references that describe chemically modified nucleic acids that were available prior to October of 1981 and were used intracellularly. Collectively, they

present uncontestable evidence that a variety of stabilized nucleic acids were known and available for use in the claimed invention as of the original filing date.

A second chemically modified nucleic acid was the methylated ribonucleic acids described by Befort *et al.* (1974). Befort is already of record as reference A27. In Befort, the authors reported uptake of their stabilized RNA into fibroblasts and the subsequent inhibition of viral multiplication. The stabilized nucleic acid was a methylated RNA that complemented a portion of the viral genome.

In Tennant *et al.* (1974), the authors describe the *in vivo* effects of an alkylated homopolymer of ribonucleic acid on virally induced oncogenesis. Tennant is already of record as reference A47.

In Kunkel *et al.* (Exhibit 1), *P.N.A.S. USA* 78(11):6734 (1981). The authors describe work conducted and published before 1981 using thio-substituted deoxynucleosides. On column 2 of page 6734, the authors describe that their analogs were previously reported as incorporated into oligonucleotides using DNA polymerase and nuclease resistant.

Finally, Miller *et al.* reported on the *in vivo* effects of a DNA analog in March 1981. This reference is already of record as A2. Attached to this declaration as Exhibit 2 is a true copy of the Medline abstract entry for this reference. The entry clearly identifies its publication date as March 1981, seven months before applicant's filing date. The analog described by Miller in 1981 was an alkyl phosphonate which differs from the phosphotriester of their earlier work by the direct attachment of the alkyl substituent to the phosphate. The Examiner is asked to review page 1879, second column, where a discussion of the intracellular half-lives of the phosphonates and the triesters are compared.

Thus, it is clear that as of the priority filing date of the present application, those of skill would have understood the applicant's reference to stabilized nucleic acid to have included more than the phosphotriester compounds that were specifically identified. Moreover, and because the use of stabilized nucleic acids was a mere example in a universe where both stabilized and natural oligonucleotides would function to downregulate expression of protein under *in vivo* conditions, it was, in my opinion, unnecessary to identify for those of skill all

the stabilized nucleic acids that were available for use in the invention as of October of 1981.

2. It is my understanding that the Examiner was concerned that the specification as filed would not have suggested that the nucleotide analogs described in the above references were useful in the invention. There are several objective reasons, why those of skill in 1981 would have understood that the text of the specification, i.e., page 4, lines 8-13 and claims 29-33 of the original specification was referring to the above identified body of knowledge.

The above referenced text of the applicant's disclosure states:

> The preferred oligonucleotide ... , for increased stability, may be transformed into a more stable form, such as a phosphotriester form, to inhibit degradation during use.

Original claims 29 and 32 recite stable forms of olignucleotides that inhibit degradation by organisms and claims 30 and 33 recite phosphotriester forms. The applicant clearly is teaching that stable, nuclease resistant forms of nucleic acid which can duplex to target nucleic acid are preferred forms of the oligonucleotides useful in the invention. Even presuming that those of skill were unaware of the above body of knowledge, to the best of my knowledge, there was no other body of knowledge to which the applicant could have been referring.

Having explained that there was no other body of knowledge that might have been confused with the above reference body of knowledge, it is simply a matter of establishing that one of skill would have had the skill to locate the above references. The above references are representative of a significant body of work involving stabilized nucleic acids for understanding enzyme mechanics, for transcription studies, for evaluting cellular uptake of nucleic acid, and for medical uses. For the Examiner to maintain that those of skill would not have known of the above references or not have been able to find the above references is contrary to the way scientists work and contrary to my understanding of how the Patent Office establishes obviousness. The phosphotriester reference in the original application would have only lead one of skill directly to Dr. Paul Miller's work and thus to other analogs. Dr. Miller's published work involved both phosphotriester

and phosphonates analogs. Anyone familiar with Dr. Miller's work would have known of analogous work by Dr. Fritz Eckstein using thio-substituted nucleic acid. Even undergraduates were being taught in 1981 that methylation was a key modification to nucleic acid for the purpose of increasing its half-life. In addition, the Examiner is asked to review the work of Dr. Summerton in 1978. This reference is already of record (A~~33~~ 26). At page 89, Dr. Summerton summarized the art of modified nucleic acids for inhibiting viral replication and specifically addressed degradation problems. Among the modified nucleic acids taught by Summerton as useful as *in vivo* antiviral agents were the methylated oligonucleotides, thio-substituted nucleic acids as well as the modified olignucleotides of Miller.

Finally, as one of skill in 1981, I can state unequivocaly that I would have fully understood what nucleic acids analogs were being referred to by the applicant in his original application. For the above reasons, I am of the opinion that the Examiner's concern regarding whether one of skill would have understood the applicant's teaching to be inadequate to identify other modified nucleic acids is groundless.

B. UNMODIFIED RNA AND DNA HAVE A HALF-LIFE *IN VIVO* THAT IS SUFFICIENT TO PERMIT ITS UPTAKE INTO CELLS.

The Examiner questions whether unmodified nucleic acid will actually survive under *in vivo* conditions for a sufficient length of time to actually be taken up by the cells. There are numerous studies that should convince the Examiner that his concerns are without foundation. Unmodified nucleic acid does survive in the body for a fairly long period. While its susceptibility to degradation make more stable forms of nucleic acid a preferred embodiment, susceptibility to degradation does not render unmodified nucleic acid useless. It is merely a matter of dosage with unmodified nucleic acid requiring higher amounts and/or longer administration to see the equivalent effects of stabilized nucleic acid.

While most articles report on the use of DNA, unmodified RNA will also survive under *in vivo* conditions. In Michelson *et al.* (1985) "Poly(A)·Poly(U) as Adjuvant in Cancer Treatment Distribution and Pharmacokinetics in Rabbits (42082)," *Proc. Soc. Exp. Biol. & Med.* 179:180-186 (Exhibit 3). In Michelson *et al.*, the authors describe the half-life of synthetic polyribonucleotides as measured in days. Its uptake into cells was also reported on page 184, 1st Col. A second report of long term survival of RNA appeared in Wolff *et al.* (1990) "Direct Gene Transfer into Mouse Muscle in Vivo," *Science*, 247:1465-1468 (Exhibit 4). In Michelson, purified RNA and DNA were simply injected into the muscle of mice and their respective gene products measured. The authors clearly state in their abstract that, "protein expression was detected in all cases and **no special delivery system was required....**"

Reports involving the use of purified DNA are more numerous than of RNA. Illustrative reports of DNA expression of plasmids directly injected into animals are provided in Lin *et al.* (1990) "Expression of Recombinant Genes in Myocardium In Vivo After Direct Injection of DNA," *Circulation*, 82:2217-2221 and Wolff *et al.* (1992) "Long-Term Persistence of Plasmid DNA and Foreign Gene Expression in Mouse Muscle," *Human Mol. Genet.* 1(6):363-369 which are Exhibits 5 and 6, respectively. The DNA may be linear or circular (see Exhibit 4 at page 368, 2nd Col.)

C. UNMODIFIED ANTISENSE OLIGONUCLEOTIDES HAVE BEEN DEMONSTRATED TO BE USEFUL IN A VARIETY OF DIFFERENT ORGANS.

Naked, natural phosphodiester, antisense oligodeoxynucleotides have been reported as sufficiently stable to downregulate gene expression when directly injected into an animal. For example, Phillip *et al.* (1994) "Antisense Inhibition of Hypertension: A new strategy for Renin-Angiotensin Candidate Genes," *Kidney Intern.*, 46:1554-1556 (Exhibit 7) reports on the direct injection of an antisense DNA (unmodified) for reducing hypertension in mice. The DNA was merely injected into the mouse carotid artery using a saline solution.

Others have reported that antisense DNA will work when directly injected into the brain. For example, in Akabayashi *et al.* (1994) *Mol. Brain Res. 21:55-61*, the authors dissolved the antisense DNA in saline and simply injected it into the brain to inhibit production of a neuropeptide (Exhibit 8). At page 56, 1st Col., the authors state that theirs is the third such report.

As stated above, the use of stabilized DNA was merely a preferred embodiment. The use of unmodified DNA was less preferred, but similar results could be achieved by merely using more DNA or RNA to accommodate instability. Given the level of skill of those practicing molecular biology, this is an intuitively apparent solution to an obvious problem. The use of high levels of DNA is described in Exhibit 9, Hijya *et al.* (1994) *Proc. Natl. Acad. Sci., U.S.A.* 91:4499-4503. Hijya *et al.* report on the use of an unmodified phosphodiester oligonucleotide for controlling the expression of a gene which is involved in skin cancer. The authors applied the antisense oligonucleotide via a subcutaneous route and used constant-infusion pumps to ensure that the oligonucleotide was adequately administered.

D. THERE IS NO UNDUE EXPERIMENTATION INVOLVED IN THE ADMINISTRATION OF ANTISENSE OLIGONUCLEOTIDES.

The level of skill of those in the art of antisense technology is quite high. Most of the artisans are like myself and hold doctorates in a relevant biological science. To achieve a measurable downregulation of protein expression, one need only contact the target cells with an adequate amount of antisense oligonucleotides. The infusion techniques are conventional and were fully known in 1981. The technique is merely the injection of a saline solution containing the antisense oligonucleotides into the appropriate organ. There is simply no basis to conclude that such a experimental step was anything but routine and intuitively apparent to those of skill.

In summary, the relative stability of unmodified antisense oligonucleotides compared to stabilized oligonucleotides does not render the *in vivo* use of unmodified DNA or RNA without utility for the purpose of downregulating protein expression. The attached references, Exhibits 3-9, clearly document to one of skill that the claimed methods are operable under *in vivo* conditions. Furthermore, there is nothing beyond routine experimentation required to administer the antisense oligonucleotides under *in vivo* conditions and detect a downregulation in the expression of a specific protein.

The declarant has nothing further to state.

Dated: April 6, 1995 By: Dennis E. Schwartz

Dr. Dennis E. Schwartz

Attachments: Exhibits 1-9

DOCKET NO.: ISIS-4502

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of : Richard H. Tullis

Appln. Serial No. : 08/078,768 Group No. : 1633

Filing Date : June 16, 1993 Examiner : J. Martinell

For : OLIGONUCLEOTIDE THERAPEUTIC AGENT AND METHODS OF MAKING SAME

DATE OF DEPOSIT: June 17, 2002
I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS FIRST CLASS MAIL, POSTAGE PREPAID ON THE DATE INDICATED ABOVE AND IS ADDRESSED TO THE COMMISSIONER FOR PATENTS AND TRADEMARKS, WASHINGTON DC 20231.

JOHN W. CALDWELL
REGISTRATION NO. 28,937

DECLARATION OF DR. STANLEY T. CROOKE PURSUANT TO 37 CFR § 1.132

I, Dr. Stanley T. Crooke, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true and statements made on information or belief are believed to be true. The Exhibit attached hereto is incorporated herein by reference.

2. I am Founder, Chairman and Chief Executive Officer of Isis Pharmaceuticals. I am currently a member of the Board of Directors of Antisense Therapeutics, Limited, Toorak, Victoria, Australia; Applied Molecular Evolutions, Inc., San Diego, California; EPIX Medical, Inc., Cambridge, Massachusetts; Idun Pharmaceuticals, Inc., La Jolla, California; and Axon Instruments, Inc., Union City, California. I also am a member of the IBC Advisory Council, Current Drugs Advisory Board, the Editorial Advisory Board of Journal of Drug

Targeting and Antisense Research and Development, and the Editorial Board of Gene Therapy and Molecular Biology. I also am Editor-in-Chief of Current Opinion in Anticancer Drugs and Section Editor for Biologicals and Immunologicals for Expert Opinion on Investigational Drugs. I have been appointed by the American Association for Cancer Research to serve as a member of the California State Legislative Committee.

I am an adjunct professor at University of California, San Diego and San Diego State University. I have authored over 425 publications and have edited 19 books.

Prior to founding Isis Pharmaceuticals, I was President of Research and Development for SmithKline Beckman Corporation (SKB). Prior to joining SKB, I helped establish the anticancer drug discovery and development program at Bristol Myers.

A copy of my *curriculum vitae* is attached as Exhibit 1.

3. As early as 1970, I worked with oligonucleotides and their uptake by cells. As early as 1971, I co-authored scientific journal articles regarding the same. In 1972, I published my doctoral thesis entitled "Preliminary studies on genetic engineering: The uptake of oligonucleotides and RNA by Novikoff hepatoma ascites cells." I was familiar at that time with phosphorothioate oligonucleotides. Indeed, in my doctoral thesis I referred to the work of DeClercq as disclosing, *inter alia*, increased stability of polynucleotides to RNAse rendered by "methods such as thiophosphate substitution." It was routine for me to refer to the works cited in a scientific article and to perform literature searches to provide the background for my research.

A routine literature search by one of ordinary skill in the art would have yielded a number of stabilized oligonucleotides, including phosphorothioates, alkylphosphonates, and phosphotriesters, available to those skilled in the art as of October, 1981.

4. I have read and am familiar with the contents of the above-referenced patent application. I have read and agree with the August 19, 1994 and April 14, 1995 declarations of Dr. Jerry L. Ruth and Dr. Dennis H. Schwartz. I further understand that the nature of the rejection at issue in the pending application is that the Examiner believes that the pending claims should be restricted to phosphotriester-modified nucleic acids which are included in the specification as a preferred embodiment as representing a class of stabilized oligonucleotides for *in vivo* applications. The Examiner alleges that the pending claims, calling for stabilized oligonucleotides, are overbroad.

The purpose of this declaration is to address this issue. In particular, I will explain that: (1) as of the priority date, one of ordinary skill in the art was aware of the existence of stabilized forms of oligonucleotides in addition to phosphotriesters and would have been guided by the disclosure of the application to the other stabilized oligonucleotides that are suitable, along with phosphotriester oligonucleotides, for *in vivo* use according to the claimed invention; (2) nothing more than routine experimentation is involved in determining which forms of stabilized oligonucleotides will work in the invention; (3) the stabilized oligonucleotides known in the art as of October 23, 1981 are taken up by cells, are sufficiently stable to exert biological activity, and specifically hybridize to their target mRNAs ; and (4) *in vitro* models correlate to *in vivo* biological activity of the stabilized oligonucleotides, as substantiated by pre- and post-filing references.

5. The application provides adequate guidance to one of ordinary skill in the art of the stabilized oligonucleotides known in the art and available prior to the October 23, 1981 filing date of the parent application.

a. Issue: The selection of the form of stabilization of the oligonucleotides for use in the invention is an inventive principle thereof and is not recited in the application.

The Facts: The point of novelty of the invention is not the form of stabilized oligonucleotides used therein. Indeed, various forms of stabilized oligonucleotides were known in the art in 1981, as discussed below. An inventive principle of Applicant's discovery is that protein expression may be specifically inhibited by targeting the coding region of a mRNA with an oligonucleotide of greater than about 14 nucleotides that is substantially complementary to the mRNA.

b. Issue: The application fails to guide those of skill in the art as to which oligonucleotides to use, as a literature search is not expected of one of skill in the art. The "scant statements" in the application are insufficient guidance as to which oligonucleotides to use in the invention.

The Facts: Stabilized oligonucleotides were known in the art in October 1981, and the application provides guidance as to which oligonucleotides to use. The language of the application made evident to one of ordinary skill in the art in 1981 that phosphotriester oligonucleotides were only a representative example of other forms of stabilized

oligonucleotides, all of which would have been equally suitable for use in the methods of the invention. To identify those other stabilized oligonucleotides, one of ordinary skill in the art need only begin with the application itself as the phosphotriester oligonucleotides set forth in the application as an example of stabilized oligonucleotides were described in the literature cited in the application. Specifically, the application cites Miller *et al.* (Biochemistry, 16:1988 (1977) ("Miller 1977")) for describing the stabilized, nuclease resistant phosphotriester form of oligonucleotides used in the examples. Thus, I am in agreement with the earlier-filed declarations of Dr. Ruth and Dr. Schwartz that the phosphotriester reference in the original application would have "lead one of skill directly to Dr. Paul Miller's work" and that "anyone familiar with Dr. Miller's work would have known of analogous work by Dr. Fritz Eckstein using thio-substituted nucleic acid." This is true because Miller et al. (Biochemistry, 13(24): 4887-4906 (1974)), referenced in the Miller 1977 article named in the application, specifically cites to the work of Eckstein.

It is my experience as a researcher that when reviewing a scientific article, one routinely refers to the works cited therein to provide the background against which the research is presented. Thus, the study of one scientific article routinely leads me to the review of several other such scientific articles.

Moreover, a routine literature search by one of ordinary skill in the art would have yielded a number of stabilized oligonucleotides available to those of ordinary skill in the art in October of 1981 and suitable for use in the invention. For example, U.S. Patent No. 3,687,808 to Merigan et al. describes stabilized phosphorothioate oligonucleotides available as early as 1972. In addition, Matzura and Eckstein (Eur. J. Biochem., 3: 448-452 (1968)) describe the nuclease resistance of phosphorothioate oligonucleotides. Agarwal and Riftina (Nuc. Acids Res., 6:9, 3009-3024 (1979)) describe the synthesis of oligonucleotides containing methyl and phenylphosphonate linkages. DeClercq et al. (Virology, 42:421-428 (1970)) describe the resistance of thiophosphate-substituted oligonucleotides to degradative enzymes. Befort et al. (Chem.-Biol. Interactions, 9:181-185 (1974) ("Befort")) report that ribonucleic acids stabilized by methylation are taken up by cells and exhibit anti-viral activity. Miller et al. (Biochem. 20(7): 1874-1880 (1981) ("Miller 1981")) report a stabilized alkyl phosphonate DNA analog having activity *in vitro*. Holý ("Synthesis and Biological Activity of Some Analogues of Nucleic Acids Components," in Phosphorus Chemistry Directed Towards Biology, W.J. Stec, Ed., Pergamon Press, 53-64, 1980) describes modified nucleotide analogs having hydroxyl-containing aliphatic chains that are stable *in vivo* and

display inhibitory and substrate activities. Harvey et al. (Biochem. 12(2):208-214 (1973) ("Harvey")) describe 5'-terminal alkyl phosphorothioate groups as protecting groups in oligonucleotide synthesis . Malkievicz et al. (Czech. Chem. Commun., 38:2953-2961 (1973) ("Malkievicz")) demonstrate the use of alkyl thioyl moieties as blocking groups in oligonucleotide synthesis.

I further agree with both Dr. Ruth and Dr. Schwartz that,

> The above references are representative of a significant body of work . . . . For the Examiner to maintain that those of skill would not have known of the above references or not have been able to find the above references is contrary to the way scientists work . . . .

April 14, 1995 declarations of Dr. Ruth and Dr. Schwartz at 4. Summerton et al. (J. Theor. Biol. 78:77-99 (1979) ("Summerton")), reviewing the modified oligonucleotides available in 1979, confirms that a person of ordinary skill in the art could have located the references describing stabilized oligonucleotides with only reasonable diligence as much as two years prior to Applicant's priority date.

Additionally, the rate of developments in the "field of chemical synthesis" of oligonucleotides in the early 1980s is irrelevant to the present analysis. That improvements to methods of chemically synthesizing oligonucleotides were being made in the early 1980s has no bearing on the patentability of the methods of selectively inhibiting the expression of a target protein in a cell without inhibiting the expression of other proteins.

Moreover, the rate of developments in the field of stabilization of oligonucleotides in the early 1980s was not so rapid, as evidenced by the broad timeframe over which the aforementioned publications became available and as further reflected by references published shortly after the 1981 priority date, that the level of skill in the art changed dramatically over the course of only a few months. For example, Miller et al. (Nucleic Acids: The Vectors of Life, 521-535 (1983) ("Miller 1983")), which was submitted for publication prior to the effective filing date, describe alkyl phosphotriester and methylphosphonate oligonucleotides as nuclease resistant analogs that are taken up by cells in culture. Likewise, Vosberg et al. (J. Biol. Chem., 257(11): 6595-6599 (1982) ("Vosberg")) and Connolly et al. (Biochem., 23(15):3443-3453 (1984) ("Connolly")) describe the nuclease resistance and resulting increased oligonucleotide stability through the use of phosphorothioate linkages.

Thus, I can state unequivocally that it is clear that, as of the October 23, 1981 priority date of the present application, those of ordinary skill in the art would have understood Applicant's reference to stabilized oligonucleotides to have included more than just the phosphotriester compounds that were specifically identified and that the application provides adequate guidance as to which stabilized oligonucleotides to use in the invention given the level of skill in the art.

6. The application provides adequate guidance to one of ordinary skill in the art as to how to use other stabilized oligonucleotides in the methods of the invention.

a. Issue: Undue experimentation would be required to practice the invention.

The Facts: Nothing more than routine experimentation was necessary to use the stabilized oligonucleotides known in the art in October 1981 in the invention. The methods for using different stabilized oligonucleotides according to the invention are essentially identical to the methods for using phosphotriester oligonucleotides set forth in the application. The stabilized oligonucleotide is simply administered, and the expression of the target protein is monitored. This experimentation was routine for one of ordinary skill in the art in 1981.

b. Issue: The application provides no guidance regarding the internalization of the stabilized oligonucleotides *in vivo*. Gura and Rojanasakul support the contention that not all stabilized oligonucleotides are internalized by cells. The post-priority date references cited by the applicant are not available to the applicant as rebuttal evidence.

The Facts: The specification provides adequate guidance regarding internalization of stabilized oligonucleotides by cells. In regard to cellular uptake of the stabilized oligonucleotides, I am in agreement with the August 19, 1994 declarations of Dr. Ruth and Dr. Schwartz. It was known in the art in 1981 that short oligonucleotides are internalized by cells in the absence of special culture conditions or methods to stimulate uptake thereof. This fact is evidenced by several pre-priority date references, including Befort, Miller 1981, and Summerton, each of which describes the uptake of modified oligonucleotides by cells. For example, the Befort article reports that "[a]ll RNAs, modified or not, enter the cells . . . ." Befort at 181. The Miller 1981 abstract states that "[t]ritium-labeled oligodeoxyribonucleoside methylphosphonates . . . are taken up intact by mammalian

cells in culture." Likewise, Summerton describes the uptake of DNA and RNA by prokaryotic and eukaryotic systems.

Moreover, I note that Gura is not a peer-reviewed article by a practicing scientist. Rather the article was written by a ***reporter* for the *Chicago Tribune***. Gura at 577. Gura merely represents a biased and untrained assessment of antisense technology by an individual having no skill in the art who solicited the ***opinions of admitted skeptics*** of the technology, including Cy Stein, Arthur Krieg, and others. Gura at 575. The news article contains no counterbalancing opinions by those who have been successful in oligonucleotide technology. The lengthy prosecution of the present application has allowed us to witness the successes achieved in the field of antisense technology, ratifying the views of proponents of antisense at the time of the invention and silencing, and indeed converting, many critics to what is clearly the correct view. ***Scientifically grounded, peer-reviewed journal articles*** support the fact that stabilized oligonucleotides are internalized by cells. These journal articles were authored by individuals of ordinary skill practicing nothing more than what is taught by the present application. In sum, Tullis was right then – true scientists knew it – and is right today.

The Rojanasakul article cited by the Examiner reports that, "[w]hen added directly to cells in culture, only 1-2% of the added [oligonucleotides] become cell-associated." Rojanasakul at 118, 120. Rojanasakul also states that there are "examples of successful in vivo treatment in the absence of specialized delivery systems." *Id.* at 118. Additionally, Rojanasakul reports that phosphorothioate oligonucleotides readily compete with unmodified oligonucleotides for cellular uptake and that methylphosphonates also enter cells. *Id.* at 120. Thus, Rojanasakul supports cellular uptake of stabilized oligonucleotides.

Additionally, Phillips et al. (Kidney International, 46: 1554-1556 (1994)) report the *in vivo* effects of phosphorothioated oligodeoxynucleotides simply injected into the brains of rats and concluded that "[o]ur present results do show that sufficient [oligodeoxynucleotide] uptake occurs in vivo to provide inhibition of blood pressure which appears to be related to the inhibition of angiotensin gene or AT_1 receptor gene expression." Phillips at 1556. Additionally, Hijiya et al. (Proc. Natl. Acad. Sci. 91: 4499-4503 (1994)) report that phosphorothioate-modified antisense oligodeoxynucleotides targeted to the MYB protooncogene controlled the growth of a human leukemia in a SCID mouse model. Thus, Phillips and Hijiya reinforce the operability of the claimed methods. Mercola et al. (Cancer Gene Therapy, 2(1): 47-59 (1995)) lends further support to the *in vivo* operability of stabilized oligonucleotides. Mercola describes several studies in which phosphorothioate

oligonucleotides administered systemically downregulate the *in vivo* expression of their targets. *See, e.g.*, Mercola at 54-55. Additionally, Putnam (Am. J. Health-Syst. Pharm. 53: 151-160 (1996)) describes several *in vivo* studies demonstrating cellular uptake of modified oligonucleotides. *See, e.g.*, Putnam at 154 ("When day-old Pekin ducklings were infected with duck hepatitis B virus and then, two weeks later, given daily intravenous injections of an antisense oligonucleotide for 10 days, there was reproducible dose-dependent inhibition of viral replication."); 156 ("In vivo studies in rats have also yielded promising results. Antisense oligonucleotides recognizing *c-myc*, cdk 2, and cdc 2 inhibited the proliferation of smooth muscle cells after carotid artery angioplasty.") (citations omitted). Thus, these post-priority date references confirm what was already known in October 1981 about the cellular uptake of stabilized oligonucleotides *in vivo*.

c. Issue: The application provides no guidance regarding the *in vivo* stability of modified oligonucleotides. The Examiner points to Gura and Rojanasakul to support the assertion that modified oligonucleotides are not stable *in vivo*.

The Facts: The Examiner relies on Gura to support his contention that modified oligonucleotides are not stable *in vivo*. Gura, however, poses the incorrect question. The appropriate question that should have been posed in that news article is not "do antisense compounds 'work the way researchers once thought they did'?" but rather "what are the pharmacokinetic data regarding the stability of modified oligonucleotides in cells and in an integrated system, such as an animal?" As presented herein, ***scientifically grounded, peer-reviewed journal articles*** support the fact that modified oligonucleotides are stable in cells. These journal articles were authored by individuals of ordinary skill practicing nothing more than what is taught by the present application.

The application provides adequate guidance regarding the stability of stabilized oligonucleotides. I am also in agreement with the April 14, 1995 declarations of Dr. Ruth and Dr. Schwartz stating that it was known in the art in October 1981 that modified oligonucleotides are stable in cells. For example, Befort reports the inhibition of Sindbis virus by chemically modified RNAs in chicken embryo fibroblasts.

Stability of modified oligonucleotides has been repeatedly confirmed by post-priority date references. Even Gura supports the stability of modified oligonucleotides *in vivo* by describing the many effects resulting from administration thereof in cell culture and *in vivo*. *See, e.g.*, Gura at 575 ("Researchers also made [the oligonucleotides] more resistant to the

many enzymes that break down nucleic acids by replacing a critical oxygen atom in each nucleotide building block with a sulfur atom."); 577 (describing the *in vivo* effects of phosphorothioate oligonucleotides). Likewise, Rojanasakul supports the *in vivo* stability of modified oligonucleotides. *See, e.g.*, Rojanasakul abstract (explaining that protected or modified oligonucleotides are stabilized against degradation in a biological environment); 119 (describing chemically modified oligonucleotides, including phosphorothioates, phosphorodithioates, and methylphosphonates, as preferred alternatives to naturally occurring phosphodiester oligonucleotides in view of *in vivo* stability). Phillips et al. (Kidney International 46: 1554-1556 (1994)) report that injections of phosphorothioated antisense oligodeoxynucleotides directed against angiotensin II type 1 mRNA into hypertensive rats produced a long lasting decrease in blood pressure. Additionally, Hijiya et al. (Proc. Natl. Acad. Sci. 91:4499-4503 (1994)) demonstrate that subcutaneous administration of phosphorothioated oligonucleotides targeting the MYB protooncogene suppressed MYB gene expression in a SCID mouse model. Mercola and Cohen (Cancer Gene Therapy 2(1): 47-59 (1995)) describe studies in which systemic delivery of modified oligonucleotides yields significant downregulation of expression of the targeted gene *in vivo*. Similarly, Putnam (Am. J. Health-Syst. Pharm. 53:151-160 (1996)) summarizes several studies in which modified oligonucleotides exhibit inhibitory effects *in vivo*. Thus, these post-priority date references confirm that which was already known to the ordinarily skilled artisan in 1981 regarding the stability of the modified oligonucleotides *in vivo*.

d. Issue: The application provides no guidance regarding the specificity of hybridization of the modified oligonucleotides *in vivo*. Gura and Rojanasakul support the contention that not all stabilized oligonucleotides bind.

The Facts: The Examiner relies on Gura to support his contention that stabilized oligonucleotides do not hybridize. Gura, however, poses the incorrect question. The appropriate question to be answered is "what are the specificity indices of stabilized oligonucleotides in cells and in an integrated system, such as an animal?" In fact, as presented herein, ***scientifically grounded, peer-reviewed journal articles*** support the fact that stabilized oligonucleotides hybridize specifically. These journal articles were authored by individuals of ordinary skill practicing nothing more than what is taught by the present application.

The application provides adequate guidance regarding the hybridization specificity of stabilized oligonucleotides. Given the representative example of a stabilized oligonucleotide in the application, only routine experimentation by one of ordinary skill in the art would be required to determine which other forms of stabilized oligonucleotides demonstrate specific hybridization *in vivo* using the application as a guide. It was known in the art in 1981 that stabilized oligonucleotides, including RNA and DNA analogs, bind specifically to their targets in cell culture. For example, Miller 1981 teaches that phosphonate analogs specifically bind intracellularly to initiation sites and tRNA binding sites of mRNA. Additionally, Befort uses artificially methylated nucleic acids to inhibit viral replication in cell culture. The Summerton reference also states that analogs and derivatives of nucleic acids function as a result of specific base pairing:

> [T]here are a growing number of reports on antiviral and/or anticancer activity of homopolyribonucleotides, analogs, and derivatives thereof, and a synthetic oligodeoxy-ribonucleotide. The general rationale for this work is that the introduction of such polymers into virally infected cells may lead to pairing between the introduced polymer and a specific viral structure of nucleotide sequence. Presumably such pairing would inhibit some critical function in the virus life cycle.

Summerton at 89.

The specificity of hybridization of stabilized oligonucleotides, including both RNA and DNA analogs, has been reinforced by a number of post-priority date publications. For example, the modified oligonucleotides of Phillips and Hijiya caused downregulation of expression of their target proteins. Mercola et al. (Cancer Gene Therapy, 2(1): 47-59 (1995)) and Putnam (Am. J. Health-Syst. Pharm., 53: 151-160 (1996)) lend further support to the *in vivo* operability of stabilized oligonucleotides. Mercola describes several studies in which phosphorothioate oligo*deoxy*nucleotides downregulate the *in vivo* expression of their targets. *See, e.g.,* Mercola at 54-55. Likewise, Putnam states that "[m]odification of the phosphodiester linkages in oligonucleotides can lend the sequences enzymatic stability without affecting their binding capacities." Putnam, abstract. The modifications referred to in Putnam include phosphorothioates, methylphosphonates, methylphosphotriesters, ethylphosphotriesters, and alkylphosphoramides. Putnam at 157, Figure 3. Thus, these post-priority date references confirm what was known to one of ordinary skill in the art in 1981.

In summary, (1) as of the priority date, one of ordinary skill in the art was aware of the existence of stabilized forms of oligonucleotides other than phosphotriesters and was guided by the disclosure of the application to the other stabilized oligonucleotides that are equally suitable as phosphotriester oligonucleotides for use according to the claimed invention; (2) nothing more than routine experimentation is involved in determining which forms of stabilized oligonucleotides will work in the invention; (3) the stabilized oligonucleotides known in the art at the effective filing date are taken up by cells, are sufficiently stable to exert biological activity, and specifically hybridize to their target mRNAs; and (4) the *in vitro* models correlate to *in vivo* biological activity of the stabilized oligonucleotides, as substantiated by pre- and post-filing date references.

I further declare that statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 6/14/02

By:
Dr. Stanley T. Crooke

SYNTHETIC POLYNUCLEOTIDES

Filed Aug. 14, 1969 2 Sheets-Sheet 1

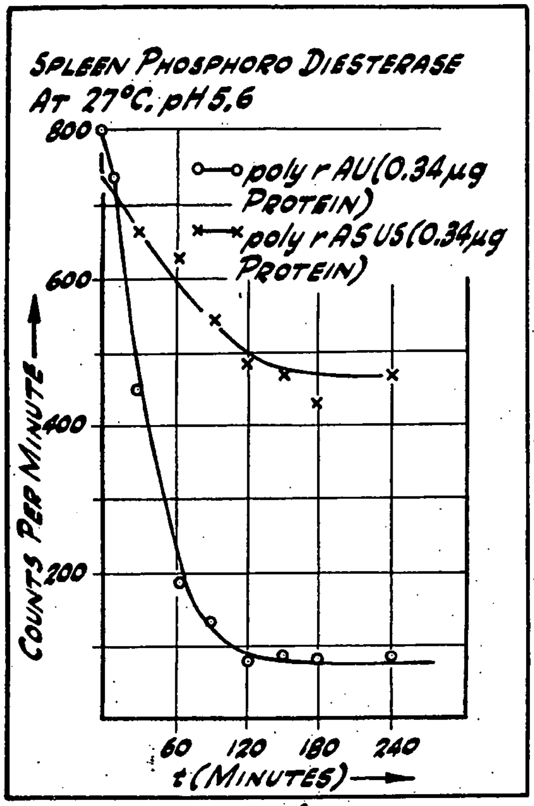

FIG. 1

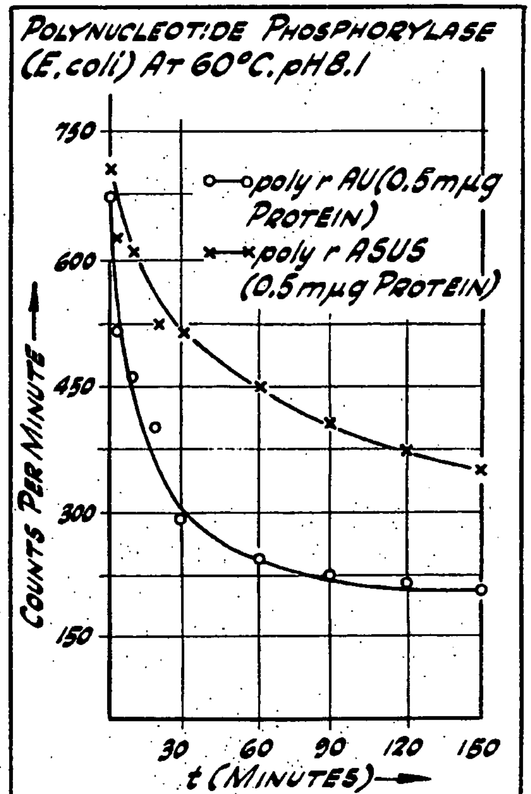

FIG. 2

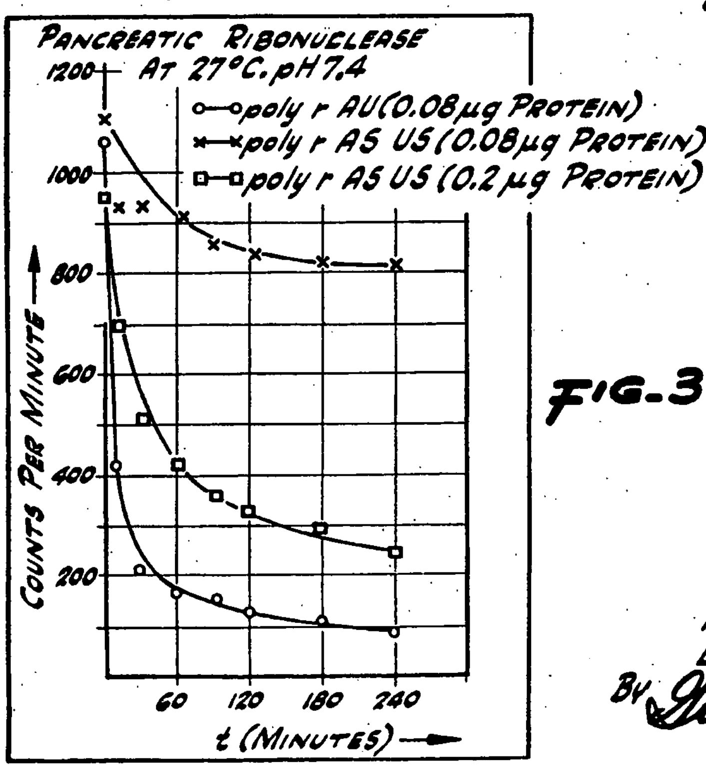

FIG. 3

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SYNTHETIC POLYNUCLEOTIDES

Filed Aug. 14, 1969 2 Sheets-Sheet 2

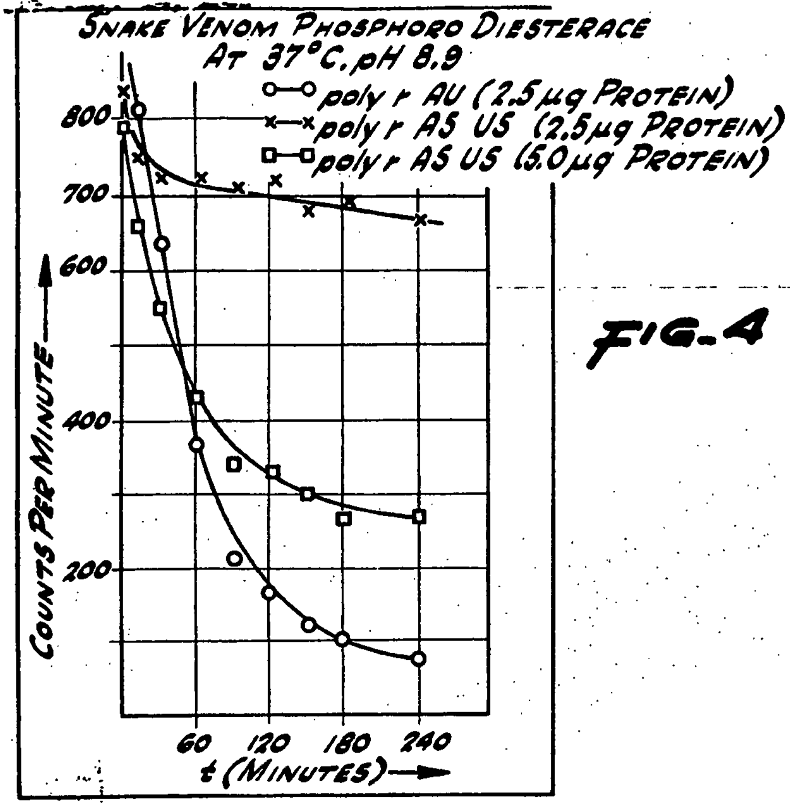

FIG. 4

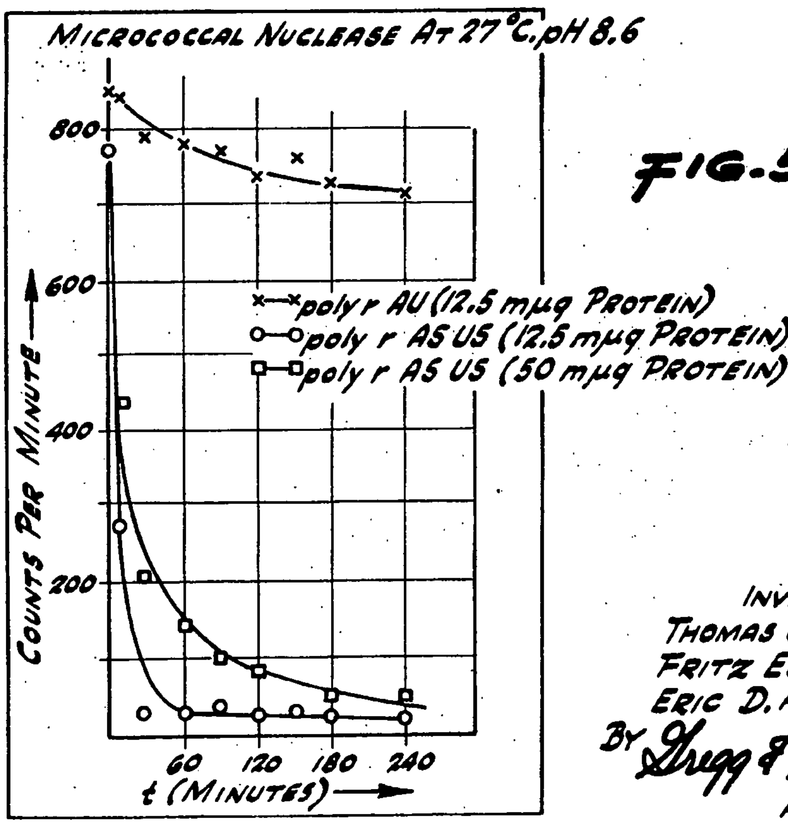

FIG. 5

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Synthesis of Oligodeoxyribonucleotide Ethyl Phosphotriesters and Their Specific Complex Formation with Transfer Ribonucleic Acid†

Paul S. Miller, J. C. Barrett,‡ and Paul O. P. Ts'o*

ABSTRACT: Oligodeoxyribonucleotide ethyl phosphotriesters, d-Tp(Et)Gp(Et)G and d-Tp(Et)Tp(Et)Cp(Et)A, were synthesized by a stepwise, chemical procedure. These triesters are complementary, respectively, to 3'-CpCpA-OH (the 3'-amino acid accepting terminus) and to -UpGpApA- (the anticodon region) of phenylalanine tRNA from yeast and *Escherichia coli*. Tritium-labeled triesters were prepared by exchange of the H-8 protons of adenine and guanine in the oligomers in tritiated water. The association constants for binding of the triesters to their complementary regions on tRNA were measured by equilibrium dialysis and were compared with those of oligodeoxyribonucleotides and oligoribonucleotides of the same sequences. In 1 M NaCl-10 mM $MgCl_2$ at 0°, the association constants of the oligomers with both $tRNA^{Phe}{}_{yeast}$ and $tRNA^{Phe}{}_{coli}$ are very similar. The association constants of the ribooligonucleotides are 8 to 20 times larger than those of the corresponding deoxyribooligonucleotides, while the deoxyribooligonucleotide triesters exhibit binding constants slightly higher than those of the deoxyribooligonucleotides. These differences are discussed in terms of the differences in conformations of the various oligomers. At low salt concentration (0.1 M NaCl, 1 mM EDTA), the oligonucleotide triesters have the same binding constants as at high salt concentration, whereas the corresponding deoxyribo- and ribooligonucleotides show a four- to sixfold decrease in their binding constants. This reflects the removal of the charge repulsion between the neutral triesters and the tRNA. The binding of oligomers to modified $tRNA^{Phe}{}_{yeast}$ was also examined. Removal of the Y base decreased the binding of anticodon-complementary oligomers sixfold while removal of the 3'-CpA residues decreased the binding of the 3'-CpCpA-OH complementary oligomers 6- to 20-fold. This study provides the chemical and physicochemical basis for the investigation of the biochemical effects of these triesters on the aminoacylation of tRNA which is reported in the following paper (Barrett, J. C., Miller, P. S., and Ts'o, P. O. P. (1974), *Biochemistry 13*, 4897).

Oligodeoxyribonucleotide alkyl phosphotriesters are oligodeoxyribonucleotide analogs containing an alkylated 3'-5' internucleotide phosphate linkage. In the preceding papers of this series (Miller *et al.*, 1971; DeBoer *et al.*, 1973; Kan *et al.*, 1973) we have shown that these oligodeoxyribonucleotide analogs have the following novel characteristics. (1) The triesters are uncharged at neutral pH. (2) Triesters form base-paired complexes with complementary polynucleotides. The complexes have a higher stability than similar complexes formed by the parent diester, presumably due to the removal of charge repulsion between the phosphate of polymer and the alkyl phosphotriester of the oligomer. (3) Trityl-containing derivatives of these compounds are very soluble in organic solvents, a feature which allowed us to investigate hydrogen-bonded, base-pairing interactions of these compounds in chloroform. (4) The methyl or ethyl groups of the dimeric phosphotriesters serve as reporters in proton magnetic resonance (pmr) studies on the conformation of the dimer, especially at the backbone region.

† From the Division of Biophysics, Department of Biochemical and Biophysical Sciences, The Johns Hopkins University, Baltimore, Maryland 21205. *Received June 24, 1974.* This work was supported in part by a grant from the National Institutes of Health (GM-16066-06) and a grant from the National Science Foundation (GB-30725X). This is paper No. 4 in a series entitled: Alkyl Phosphotriesters of Dinucleotides and Oligonucleotides. Paper No. 3 in this series is Kan *et al.* (1973).

‡ This work was submitted as partial fulfillment of the requirements for the Degree of Doctor of Philosophy to the Johns Hopkins University.

(5) The solution conformations of dinucleoside alkyl phosphotriesters are similar to those of the parent diesters, although base-stacking interactions are slightly reduced. (6) The dinucleoside alkyl phosphotriesters are chemically stable in neutral solution at room temperature and are completely resistant to hydrolysis by exonuclease enzymes.

In this paper we describe the synthesis of d-Tp(Et)Gp(Et)G[1] and d-Tp(Et)Tp(Et)Cp(Et)A[1] and their tritium-labeled derivatives. The base sequences of these triesters are complementary to the 3′-CpCpA-OH, amino acid accepting terminus of most tRNAs, and the -UpGpApA-, anticodon region of phenylalanine transfer RNA from yeast and *Escherichia coli*. These triesters were found to form hydrogen-bonded complexes with their complementary region of these tRNAs at both high and low salt concentrations. The association constants of these interactions were determined by equilibrium dialysis experiments. The results of these binding studies are compared with the results of binding studies using the comparable oligoribo- and oligodeoxyribonucleotide diesters. In the following paper the effect of triester-tRNA complex formation on the aminoacylation of tRNA is described in detail (Barrett *et al.*, 1974).

Results and Discussion

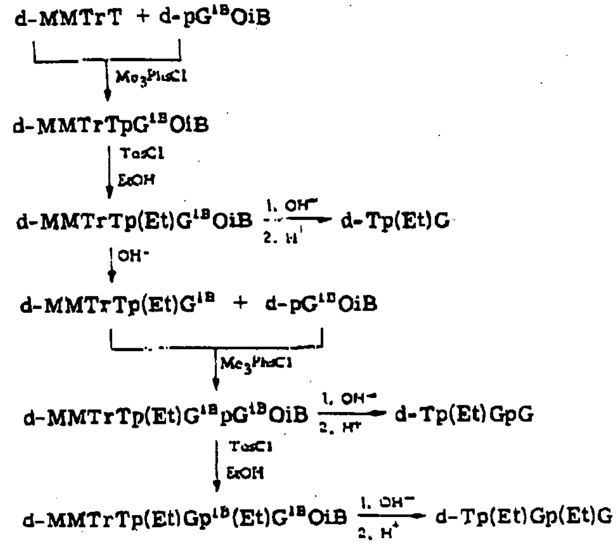
Syntheses and Stability of Oligonucleotide Ethyl Triesters. Oligonucleotide alkyl and aryl phosphotriester derivatives have been synthesized by a number of workers for use as intermediates in the syntheses of oligodeoxyribo- and oligoribonucleotides (Letsinger *et al.*, 1969; Eckstein and Rizk, 1967; Reese and Saffhill, 1968; Grams and Letsinger, 1970; van Boom *et al.*, 1971; Catlin and Cramer, 1973; Itakura *et al.*, 1973). In all cases an appropriately protected alkyl or aryl nucleoside phosphodiester was condensed with a suitably protected nucleoside or nucleoside 3′-phosphotriester to yield the triester.

In our work we have explored an approach in which the triester is formed from a suitably protected oligonucleotide phosphodiester intermediate by direct alkylation of the phosphate group (Miller *et al.*, 1971). The alkylating agent in this reaction is ethanol together with *N,N*-dimethylformamide, 2,6-lutidine, and *p*-toluenesulfonyl chloride. The tosyl chloride or its complex formed by reaction with *N,N*-dimethylformamide, $(CH_3)_2N^+{=}CHOSO_2C_6H_4CH_3$-*p* (Cl^-) (Hall, 1956), activates the phosphate group for attack by the alcohol. Cramer and Winter (1961) reported similar formation of phosphotriesters from dinucleoside phosphomonochloridates in dimethylformamide.

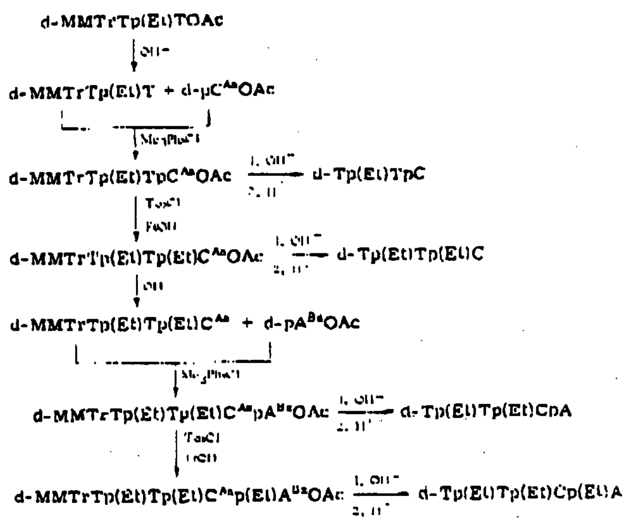
Previously Holý and Scheit (1967) have reported alkylation of the phosphate group of d-TpT using diazomethane. An 8% yield of phosphotriester was obtained, but all the thymine bases were methylated. Successful alkylation of d-TpT using diazomethane has been reported by Scheit (1967), the phosphotriester being obtained in 80% yield. More recently Nagyvary and coworkers (1973) synthesized oligothymidylate methyl triesters by the reaction of oligothymidylate with methyl methanesulfonate as the alkylating agent. The reaction resulted in an 80% phosphotriester formation, accompanied by some methylation of the thymine base.

[1] The system of abbreviations used is the same as that described by Schaller and Khorana (1963) and by van de Sande and Bilsker (1973). Np(Et)N indicates ethylation of the 3′ 5′ internucleotide phosphate linkage. Abbreviations used are: TosCl, *p*-toluenesulfonyl chloride; Me_3PhsCl, trimethylbenzenesulfonyl chloride.

Scheme I: Synthesis of d-Tp(Et)Gp(Et)G



Scheme II: Synthesis of d-Tp(Et)Tp(Et)Cp(Et)A



The synthetic routes for the preparation of d-Tp(Et)Gp(Et)G and d-Tp(Et)Tp(Et)Cp(Et)A are shown in Schemes I and II. The basic scheme involves stepwise addition of protected nucleoside 5′-phosphates to a growing oligonucleotide chain followed by ethylation of the resulting phosphodiester linkage, to give oligonucleotide triesters of a defined sequence. Thus, in the case of d-Tp(Et)Gp(Et)G (Scheme I), the protected dinucleotide, d-MMTrTpG^iBOiB, was synthesized by condensation of the appropriately protected monomers and the product was isolated and purified by extraction procedures similar to those reported by Caruthers *et al.* (1972). The diester was then ethylated. In this particular reaction an equivalent of *N*-methylimidazole was added to the ethylation media. *N*-Methylimidazole has been shown to increase the amount of phosphotriester formation in the synthesis of oligothymidylate ethyl phosphotriesters (R. Pless, personal communication). However, a fluorescent side product which appeared to result from alkylation of the guanine ring was detected in this reaction. Therefore, addition of *N*-methylimidazole was avoided in further ethylation reactions involving gua-

TABLE I: Spectral Characteristics of Oligonucleotides and Oligonucleotide Triesters in Water, pH 7.

	λ_{max} (nm)	λ_{min} (nm)	$\epsilon_{255}/\epsilon_{270}$	ϵ (λ_{max})
d-TpG[a]	255, 270 (s)	229	1.18	
d-TpGpG[b]	255, 270 (s)	230	1.24	32.5×10^3
d-Tp(Et)G	255, 270 (s)	228	1.17	
d-Tp(Et)GpG	255, 270 (s)	227	1.30	
d-Tp(Et)Gp(Et)G	255, 270 (s)	227	1.24	33.3×10^3
d-GpGpT[c]	255, 270 (s)	232	1.25	31.7×10^3
			$\epsilon_{260}/\epsilon_{280}$	
d-Tp(Et)T	267	236	1.53	
d-Tp(Et)TpC	267	237		
d-Tp(Et)Tp(Et)C	267	237	1.33	
d-Tp(Et)Tp(Et)CpA	264	234		
d-Tp(Et)Tp(Et)Cp(Et)A	263	234	1.77	35.8×10^3
d-TpTpCpA	263	235	1.80	34.2×10^3

[a] The spectral properties of this compound are similar to those of d-pTpG (Büchi and Khorana, 1972). [b] Similar spectral properties were observed by Caruthers *et al.* (1972). [c] The spectral properties of this compound are similar to those of d-TpGpG. Further characterization is given in the Experimental Section.

TABLE II: Paper Chromatography and Paper Electrophoresis of Oligonucleotides.

	R_F^A	R_F^C	R_F^F	R_F^I	R_m^a
d-TpG[b]	0.24	0.42	0.51		0.39 (dpG)
d-Tp(Et)G	0.51	0.70	0.67	0.65	−0.21 (dpT)
d-Tp(Et)GpG	0.061	0.36	0.47		0.29 (dpG)
d-TpGpG[b]	0.037	0.22	0.32		0.70 (dpG)
d-Tp(Et)Gp(Et)G	0.29	0.63	0.60	0.45	−0.17 (dpT)
d-GpGpT[c]	0.033	0.18	0.34		0.66 (dpT)
d-Tp(Et)T				0.78	0.00 (dpT)
d-Tp(Et)TpC	0.23	0.45	0.56	0.39	0.45 (dpT)
d-Tp(Et)Tp(Et)C	0.34	0.67	0.66	0.43	0.10 (dpT)
d-Tp(Et)Tp(Et)CpA	0.16	0.40	0.56	0.27	0.23 (dpT)
d-Tp(Et)Tp(Et)Cp(Et)A	0.26	0.54	0.63	0.31	0.07 (dpT)
d-TpTpCpA[d]			0.37	0.07	0.57 (dpA)

[a] Mobility relative to the indicated nucleoside 5′-phosphate at pH 8.5. The negative sign indicates that the compound moved toward the cathode, probably due to solvent movement. [b] Similar chromatographic mobilities were observed by Ohtsuka *et al.* (1972) using solvent systems A and F, and by Caruthers *et al.* (1972) using solvent system F. [c] The chromatographic and electrophoretic mobilities of this compound are similar to those of d-TpGpG. Further characterization is given in the Experimental Section. [d] The chromatographic mobility of this compound was similar to that of d-TpTpApC (Ohtsuka and Khorana, 1967). Further characterization is given in the text.

nine-containing oligomers.

The 3′-*O*-isobutyryl protecting group was selectively removed by treatment with aqueous sodium hydroxide solution. The oligonucleotide chain was then extended by addition of d-pG^{iB}OiB. The resulting diester–triester intermediate, d-MMTrTp(Et)G^{iB}pG^{iB}OiB, was readily purified by silica gel column chromatography. Ethylation then gave the fully protected phosphotriester which was also purified by silica gel column chromatography. The protecting groups were removed by hydrolysis in concentrated ammonium hydroxide solution followed by treatment with 80% acetic acid. The resulting d-Tp(Et)Gp(Et)G was purified by preparative paper chromatography. An analogous stepwise synthetic procedure was followed in the preparation of d-Tp(Et)Tp(Et)Cp(Et)A (Scheme II). The resulting oligonucleotide triesters are obtained as a mixture of diasteroisomers (Miller *et al.*, 1971). No attempt was made to separate the diastereomers.

During the syntheses, protecting groups were removed from samples of the various protected triester intermediates and the products were further characterized. For example, d-MMTrTp(Et)G^{iB}OiB gave d-Tp(Et)G. This triester has ultraviolet (uv) spectral characteristics identical with those of d-TpG (Table I) and behaves as a neutral molecule on paper chromatography and paper electrophoresis (Table II). In addition, d-Tp(Et)G is resistant to hydrolysis by both spleen and snake venom phosphodiesterase enzymes. Removal of protecting groups from the triester–diester intermediates, d-MMTrTp(Et)G^{iB}pG^{iB}OiB and d-MMTrTp(Et)Tp(Et)C^{An}pA^{Bz}OAc, gave d-Tp(Et)GpG and d-Tp(Et)Tp(Et)CpA, respectively. These oligomers have uv spectral characteristics similar to those of the diesters d-TpGpG and d-TpTpCpA (Table I). Their paper electrophoretic mobilities indicated that these oligomers con-

TABLE III: Tritium-Labeled Oligodeoxyribonucleotides.

	cpm/A_{max} Unit	Sp Act. (Ci/mol)[a]	% Exchange
[^{3}H]d-Tp(Et)Gp(Et)G	1.14×10^6	45	52
[^{3}H]d-TpGpG	1.07×10^6	41	49
[^{3}H]d-Tp(Et)Tp(Et)Cp(Et)A	2.67×10^5	11	26
[^{3}H]d-TpTpCpA	1.86×10^5	7.6	18

[a] Counting efficiency, 38%.

tain only one charge (Table II). Treatment of d-Tp(Et)GpG with snake venom phosphodiesterase, an exonuclease which requires a free 3'-hydroxyl group and proceeds in a 3' to 5' direction, gave d-Tp(Et)G and d-pG as products. Similar treatment of d-Tp(Et)Tp(Et)CpA gave d-Tp(Et)Tp(Et)C and d-pA. Both oligomers were resistant to hydrolysis by spleen phosphodiesterase, an exonuclease which requires a free 5'-hydroxyl group and proceeds in a 5' to 3' direction.

In general the oligonucleotide ethyl phosphotriesters are stable to hydrolysis in neutral, aqueous solutions and have been stored for periods of months under these conditions. The ethyl phosphotriester linkage is stable in 0.1 M hydrochloric acid and in 0.1 M sodium hydroxide solutions for at least 24 hr at 28°. In addition, this triester linkage was found to be stable to hydrolysis in concentrated ammonium hydroxide solutions for at least 2 days and in 1 M sodium hydroxide for 10 min at room temperature. These various pH conditions are often encountered in deprotecting oligonucleotides during synthetic reactions.

While the triester linkage is resistant to snake venom and spleen phosphodiesterase, Dudman and Zerner (1973) recently reported a phosphotriesterase activity in mammalian sera. We found that oligonucleotide phosphotriesters are completely stable to hydrolysis when incubated in 10% fetal calf serum or 10% human cord serum at 37° for up to 139 hr. Under the same conditions the corresponding oligonucleotide diesters are hydrolyzed to their component monomers. The stability of the phosphotriesters under these conditions allows them to be used in biological studies on mammalian cells in culture.

Interaction of Oligonucleotide Triesters with tRNA. The interaction of the oligonucleotide triesters with tRNA^{Phe} from yeast and *E. coli* was studied by equilibrium dialysis techniques. These studies required that the triesters contain a tritium label of relatively high specific activity. The triesters, d-Tp(Et)Gp(Et)G and d-Tp(Et)Tp(Et)Cp(Et)A, and the corresponding diesters were labeled by heating the oligomers in tritiated water at 90° to exchange the H-8 proton in the purine ring (Ts'o *et al.*, 1969). After removal of excess tritiated water by lyophilization, the labeled oligomers were purified by paper chromatography. As shown in Table III, this procedure gave oligomers of sufficiently high specific activity for use in the dialysis experiments.

The results of experiments measuring the interaction of the oligodeoxyribonucleotide triesters and their parent diesters with tRNA$^{Phe}{}_{yeast}$ and tRNA$^{Phe}{}_{coli}$ are presented in Table IV. These studies were carried out at 0° in a buffer containing 10 mM $MgCl_2$, 10 mM Tris (pH 7.5), and either 1 M NaCl or 0.1 M NaCl. The latter condition approximates the ionic strength conditions used in the *in vitro* aminoacylation of tRNA. The tRNA concentration was 40 μM. The association constants were determined directly by measurement of the ratio of tritium counts found on each side of the dialysis membrane as described by Uhlenbeck (1972). In the case of d-GpGpT, which did not contain a radioactive label, the binding constants to tRNA^{Phe} were determined by competition with GpGpU (Uhlenbeck, 1972). Competition studies with GpGpU were also carried out for the oligomers d-Tp(Et)Gp(Et)G and d-TpGpG. The same results were obtained as those obtained by direct measurement.

For purposes of comparison, the ribooligonucleotides UpUpC, UpUpCpA, GpGpU, and UpGpG were also synthesized and their association constants to tRNA$^{Phe}{}_{yeast}$ were measured. Similar measurements on the binding of these oligoribonucleotides to tRNA$^{Phe}{}_{yeast}$ have been made independently by Pongs *et al.* (1973) and by Cameron and Uhlenbeck (1973). Their studies were carried out under essentially the same conditions of salt concentration, temperature, and tRNA concentration as the present investigation.

TABLE IV: Binding of Oligomers to tRNA$^{Phe}{}_{yeast}$ and tRNA$^{Phe}{}_{coli}$ at 0°.

	K (M^{-1})		
	tRNA$^{Phe}{}_{yeast}$; 1 M NaCl[a]	tRNA$^{Phe}{}_{coli}$; 1 M NaCl[a]	tRNA$^{Phe}{}_{coli}$; 0.1 M NaCl[a]
d-Tp(Et)Tp(Et)Cp(Et)A	3,000	3,600	2,400
d-TpTpCpA	2,200	2,400	1,300
d-Tp(Et)Gp(Et)G	2,400	1,700	2,000
d-TpGpG	600	1,100	500
d-GpGpT	12,900[b]	12,000[b]	11,200[b]
UpUpC	1,600 (2,000)[c]	2,000	2,300
UpUpCpA	48,000 (75,000)[c] (13,700)[d]	48,500	45,000
UpGpG	4,800 (4,500)[c]	7,300	4,900
GpGpU	224,000 (30,000)[c] (117,000)[d]	219,000	204,000

[a] The buffer also contained 10 mM $MgCl_2$–10 mM Tris (pH 7.5). [b] Measured by competition with GpGpU. [c] Pongs *et al.*, 1973. [d] Cameron and Uhlenbeck, 1973.

While the results are basically similar to each other there are some discrepancies in the association constants measured for UpUpCpA and GpGpU in the three studies. The different values obtained may reflect differences in the state of purity or the conformational state of the tRNAs used in these three studies.

The results in Table IV show that the oligodeoxyribonucleotide phosphotriesters form complexes with both $tRNA^{Phe}_{yeast}$ and $tRNA^{Phe}_{coli}$. Indeed the association constants observed for d-Tp(Et)Gp(Et)G and d-Tp(Et)Tp(Et)Cp(Et)A are slightly higher than those of the corresponding parent diesters, d-TpGpG and d-TpTpCpA. Thus, alkylation of the sugar-phosphate backbone does not interfere with binding of the triesters to the tRNA. These results are similar to those of our previous study on the 2U:1A complexes formed between poly(U) and phosphotriester derivatives of d-ApA (Miller *et al.*, 1971). Complexes of poly(U) with d-Ap(Et)A and d-Ap(Me)A have higher melting temperatures than the complex formed with d-ApA.

A significant difference is observed between the association constants for the oligoribonucleotides and the analogous oligodeoxyribonucleotides. The association constants of the oligoribonucleotides are eight to twenty times larger than those of the oligodeoxyribonucleotides. This difference may reflect the difference in conformations of the oligoribonucleotides *vs.* the conformations of the oligodeoxyribonucleotides. Our laboratory has previously shown (Kondo *et al.*, 1972) for the purine-containing dimers, d-ApA and ApA, that the extent of base–base overlap in the deoxydimer is much greater than that in the ribodimer. In addition, the extent of base–base overlap in a stacked dimer is much greater than that in a 10- to 11-fold helix. Thus in the present series, the deoxyoligomers must first reduce the extent of base–base overlap before they are in a suitable conformation to hydrogen bond with their complementary region of the tRNA. Compared to the corresponding ribooligomers, which have a lesser extent of base–base overlap in their stacking mode, the complexes formed with the deoxyoligomers would have a lower stability than the complexes formed with ribooligomers. A more rigorous discussion of this subject was presented in a recent paper concerning the effects of C-2′ substituents on polynucleotide conformation (Alderfer *et al.*, 1974). Therefore the association constant would also be expected to be lower for the oligodeoxyribonucleotide triester, since their conformations and extents of stacking are very similar to those of the corresponding oligodeoxyribonucleotide diesters (Miller *et al.*, 1971).

Table IV shows that the association constants for the various oligomers to both $tRNA^{Phe}_{yeast}$ and $tRNA^{Phe}_{coli}$ are similar. Since these oligomers serve as probes of the secondary structure of the anticodon and 3′-ApCpCpA-OH terminus of tRNAs in these binding studies (Uhlenbeck *et al.*, 1970), our results suggest that these regions in $tRNA^{Phe}_{yeast}$ and $tRNA^{Phe}_{coli}$ have very similar conformations.

The specificity of binding was investigated by studying the interaction of the triester oligomers with modified $tRNA^{Phe}_{yeast}$ and with crude $tRNA_{coli}$. The $tRNA^{Phe}_{yeast}$ was modified by treatment with dilute hydrochloric acid to remove the Y base from the anticodon loop (Thiebe and Zachau, 1970). The $tRNA^{Phe}_{yeast}$ was also modified by treatment with snake venom phosphodiesterase to remove the 3′-CpA residues from the amino acid accepting terminus (Simsek *et al.*, 1973). Recently Cameron and Uhlenbeck (1973) and Pongs and Reinwald (1973) have demonstrated that removal of the Y base from $tRNA^{Phe}_{yeast}$ causes a seven- to eightfold reduction in the association constant of UpUpCpA for the modified tRNA. As shown in Table V, we observe five- and sixfold reduction in the binding of d-Tp(Et)Tp(Et)Cp(Et)A and UpUpCpA, respectively, to $tRNA^{Phe}_{yeast}$ − Y. It is interesting to note that the binding of d-Tp(Et)Gp(Et)G and UpGpG to this tRNA was also decreased slightly.

The association of d-Tp(Et)Gp(Et)G and UpGpG with snake venom phosphodiesterase treated $tRNA^{Phe}_{yeast}$ was reduced 14- and 19-fold, respectively, when compared to binding with intact $tRNA^{Phe}_{yeast}$. The small amount of residual binding observed may be due to binding to some tRNA molecules from which only the terminal A residue has been removed. A reduction in the binding of d-Tp(Et)Tp(Et)Cp(Et)A and UpUpCpA to this modified tRNA was also observed.

The results of these experiments indicate that removal of the Y base not only disrupts binding of the oligomers to the anticodon region, but also affects binding at the more remote 3′ terminus. Similarly, removal of the 3′-CpA residues not only eliminates binding of the oligomers complementary to this region, but also reduces the binding constants of oligomers complementary to the anticodon region. This effect may result from a conformational change in the entire tRNA molecule induced by removal of the Y base or the 3′-CpA terminus.

The triester d-Tp(Et)Tp(Et)Cp(Et)A is complementary to the anticodon region of $tRNA^{Phe}$, while the triester d-Tp(Et)Gp(Et)G is complementary to the 3′-CpCpA terminus, a sequence common to all tRNAs. Therefore, the apparent association constants for binding of d-Tp(Et)Tp(Et)Cp(Et)A should be greatly reduced if a crude mixture of tRNAs is used instead of pure $tRNA^{Phe}$. However, the magnitude of binding of d-Tp(Et)Gp(Et)G to crude tRNA should be similar to the association constant for binding to

TABLE V: Binding of Oligomers to Modified tRNAs.[a]

	K (M^{-1})			K_{app} (M^{-1})
	$tRNA^{Phe}_{yeast}$	$tRNA^{Phe}_{yeast}$ − Y	$tRNA^{Phe}_{yeast}$ − CA	$tRNA_{coli}$[b]
d-Tp(Et)Tp(Et)Cp(Et)A	3,000	600	900	300
UpUpCpA	48,000	8200	29,100	1100
d-Tp(Et)Gp(Et)G	2,400	1200	170	2400
UpGpG	4,800	3400	250	9900

[a] 1 M NaCl, 10 mM $MgCl_2$, 10 mM Tris (pH 7.5); 0°; tRNA concentration 40 μM. [b] Unfractionated.

$tRNA^{Phe}$. The results of binding experiments using a mixture of crude tRNA from *E. coli* are shown in Table V. This mixture contains approximately 3.4% $tRNA^{Phe}$ as determined by aminoacylation with phenylalanine. The apparent binding constants of d-Tp(Et)Tp(Et)Cp(Et)A and UpUpCpA to crude $tRNA_{coli}$ are greatly reduced compared to their binding to $tRNA^{Phe}{}_{coli}$ (see Table IV). As predicted, the binding constants of d-Tp(Et)Gp(Et)G and UpGpG to the crude $tRNA_{coli}$ are almost the same as those obtained for binding to $tRNA^{Phe}{}_{coli}$ (Table IV). The above results (Table V) demonstrate that d-Tp(Et)Tp(Et)Cp(Et)A binds specifically with the anticodon region of $tRNA^{Phe}$ while d-Tp(Et)Gp(Et)G interacts specifically with the 3'-CpCpA terminus of all tRNA. This specificity of interaction is further demonstrated by the aminoacylation experiments described in the following paper (Barrett *et al.*, 1974).

The association constants for binding of the triesters d-Tp(Et)Gp(Et)G and d-Tp(Et)Tp(Et)Cp(Et)A to $tRNA^{Phe}{}_{yeast}$ show little or no change when the salt concentration is reduced from 1 M NaCl with Mg^{2+} to 0.1 M NaCl without Mg^{2+} (Table VI). In contrast, the association constants of the diesters, d-TpTpCpA, UpUpCpA, and UpGpG, are significantly reduced when the salt concentration is lowered. A similar effect on the magnitude of the association constant for binding of UpApCpA to $tRNA^{Tyr}{}_{coli}$ in 1 mM EDTA-10 mM Na_2HPO_4 at 0° was reported when the sodium chloride concentration was decreased from 1.5 to 0.2 M (Uhlenbeck, 1972). This decrease in binding is attributed to an increase in charge repulsion between the negatively charged sugar-phosphate backbone of the oligonucleotide diester and the tRNA as the ionic strength of the solution is lowered. Since the oligonucleotide triesters are neutral molecules, such charge repulsion is eliminated and changes in ionic strength do not significantly affect the binding constants.

The stability of the oligodeoxyribonucleotide triesters and their ability to form specific complexes with complementary regions of tRNA make these compounds attractive candidates as probes of the structure and function relationships of nucleic acid, both *in vitro* and in living cells. The effect of these triesters on the *in vitro* aminoacylation of tRNA is the subject of the following paper (Barrett *et al.*, 1974).

TABLE VI: Effect of Salt Concentration on the Binding of Oligomers to $tRNA^{Phe}{}_{yeast}$ at 0°.[a]

	K (M^{-1})	
	1 M NaCl–10 mM $MgCl_2$	0.1 M NaCl–1 mM EDTA
d-Tp(Et)Tp(Et)Cp(Et)A	3,000	3,400
d-Tp(Et)Gp(Et)G	2,400	2,500
d-TpTpCpA	2,200	0
UpUpCpA	48,000	14,800
UpGpG	4,800	800

[a] Each buffer contained 10 mM Tris (pH 7.5). The tRNA concentration was 40 μM.

Experimental Section

Chemical Synthesis of Oligodeoxyribonucleotides and Oligodeoxyribonucleotide Phosphotriesters

General. The deoxynucleosides and deoxynucleoside 5'-phosphates obtained commercially were checked for purity by paper chromatography. 5'-*O*-Mono-*p*-methoxytritylthymidine (Schaller *et al.*, 1965), 3'-*O*-acetylthymidine 5'-phosphate (Khorana and Vizsolyi, 1961), $N^2,O^{3'}$-diisobutyryldeoxyguanosine 5'-phosphate (Weber and Khorana, 1972), N^4-*p*-anisoyl-3'-*O*-acetyldeoxycytidine 5'-phosphate (Schaller and Khorana, 1963), and N^6-benzoyl-3'-*O*-acetyldeoxyadenosine 5'-phosphate (Weiman *et al.*, 1963) were prepared by methods described in the literature. 2,4,6-Trimethylbenzenesulfonyl chloride (Me_3PhSCl)[1] from Aldrich Chemical Co. and *p*-toluenesulfonyl chloride (TosCl)[1] from Eastman Chemical Co. were each recrystallized from pentane before use. Anhydrous pyridine and *N,N*-dimethylformamide were prepared according to Miller *et al.* (1971). Anhydrous ethanol was prepared by distillation from magnesium ethoxide (Shirley, 1951) onto molecular sieves (Linde Type 3A). 2,6-Lutidine was dried over molecular sieves.

Descending paper chromatography was performed on Whatman No. 3MM paper using the following solvent systems: solvent A, 2-propanol–concentrated ammonium hydroxide–water (7:1:2, v/v); solvent C, 1 M ammonium acetate–95% ethanol (3:7, v/v; pH 7.5); solvent F, 1-propanol–concentrated ammonium hydroxide–water (50:10:35, v/v); solvent I, 2-propanol–water (7:3, v/v). Paper electrophoresis was carried out on a Savant flat plate apparatus using 0.05 M triethylammonium bicarbonate as buffer (pH 8.5) at a voltage of 40 V/cm for 45 min. Ultraviolet spectra were recorded on a Cary 14 uv spectrophotometer. Unless otherwise noted, all reactions and operations were carried out at room temperature.

General Condensation Procedure. The protected nucleoside or the pyridinium salts of the oligonucleotide and protected nucleoside 5'-phosphate were dried by repeated evaporations with anhydrous pyridine. The resulting gum was dissolved in dry pyridine; the solution was cooled to 0°; mesitylenesulfonyl chloride was added and the reaction mixture was kept in the dark at room temperature. These operations were carried out in a dry nitrogen atmosphere. The reaction was terminated by addition of an equal volume of water at 0°, and, after storage (usually overnight) at room temperature, the reaction mixture was diluted with water and the products were purified by extraction and column chromatography. The isolated products were precipitated either from pyridine solution by dropwise addition to anhydrous ether for the diesters or from tetrahydrofuran solution by addition to hexane for the triesters. The precipitates were collected by centrifugation, thoroughly washed with solvent, and dried under vacuum to give amorphous, white powders. The spectral characteristics of the protected oligomers are given in Table VII.

General Ethylation Procedure. The protected oligomer diester was made anhydrous by repeated evaporation with anhydrous pyridine and the residue was dissolved in a solution containing *N,N*-dimethylformamide and 2,6-lutidine. Ethanol and *p*-toluenesulfonyl chloride (TosCl) were added at 0° and after 1 hr at room temperature an additional amount of ethanol and TosCl was added. These operations were carried out in a dry nitrogen atmosphere. The reaction was terminated after 2 hr total time by addition of water. The solvents were evaporated with frequent additions of 50% chloroform–methanol to aid in the removal of the dimethylformamide, and the residue was dissolved in chloroform. After extraction with water, the chloroform solution

TABLE VII: Spectral Characteristics of Protected Oligonucleotides and Triesters in Methanol.

	λ_{max} (nm)	λ_{min} (nm)	$\epsilon_{260}/\epsilon_{280}$
d-MMTrTpG^{iB}OiB	260, 230 (s), 277 (s)	234	1.44
d-MMTrTpG^{iB} [a]	260, 233 (s), 277 (s)	243	1.40
d-MMTrTp(Et)G^{iB}OiB	237, 263, 255 (s), 280 (s)	227, 243	1.38
d-MMTrTp(Et)G^{iB}	234, 262, 255 (s), 280 (s)	226, 243	1.34
d-MMTrTp(Et)G^{iB}pG^{iB}OiB	233, 258, 277 (s)	227, 238	1.41
d-MMTrTp(Et)G^{iB}p(Et)G^{iB}OiB	258, 237, 280 (s)	227	1.40
d-MMTrTp(Et)TOAc	267, 225 (s)	240	
d-MMTrTp(Et)TpC^{An}OAc	270, 305 (s)	242	1.00
d-MMTrTp(Et)Tp(Et)C^{An}OAc	270, 305 (s)	243	0.95
d-MMTrTp(Et)Tp(Et)C^{An}pA^{Bz}OAc	278, 315 (s)	245	
d-MMTrTp(Et)Tp(Et)C^{An}p(Et)A^{Bz}OAc [b]	278, 315 (s)	244	0.79

[a] Similar spectral properties were observed by Caruthers *et al.* (1972). [b] The spectral properties of this compound are similar to those of d-MMTrTpTpA^{Bz}pC^{An} (Ohtsuka and Khorana, 1967).

was dried over anhydrous sodium sulfate. The solution was then concentrated and the products were isolated by silica gel column chromatography. The products were precipitated from a tetrahydrofuran solution by addition to hexane. The spectral characteristics of the protected oligonucleotide triesters are presented in Table VII.

Removal of Protecting Groups. The 3′-*O*-protected oligomer was dissolved in 50% ethanol–pyridine and treated with an equal volume of 2 N sodium hydroxide for 7 min at −5° (3′-*O*-isobutyryl group) or 15 min at room temperature (3′-*O*-acetyl group). The reaction mixture was then neutralized with pyridinium Dowex 50X resin. The resin was filtered and thoroughly washed with 50% ethanol–pyridine and the combined filtrate and washings were evaporated. The products were isolated by precipitation.

For complete removal of protecting groups, the protected oligomer was first treated with 60% concentrated ammonium hydroxide in pyridine for 2–3 days. After evaporation of solvents the residue was treated with 80% acetic acid for 3–6 hr. The acetic acid was removed by evaporation. Spectral characteristics and mobilities on paper chromatography and paper electrophoresis of deblocked oligomers are presented in Tables I and II, respectively.

Hydrolysis with Snake Venom and Spleen Phosphodiesterase. The previously published procedure (Miller *et al.*, 1971) was followed.

Preparation of d-MMTrTpG^{iB}OiB and d-TpG. d-MMTrT (5.15 g, 10 mmol) and d-pG^{iB}OiB (6.25 g, 11 mmol) were allowed to react for 4 hr in 50 ml of pyridine containing Me_3PhsCl (5.26 g, 24 mmol). After overnight aqueous pyridine treatment, the reaction mixture dissolved in water (200 ml) was extracted with ether (2 × 600 ml). Additional water (700 ml) was added and the solution was extracted with 1-butanol (2 × 750 ml). The combined butanol extracts were evaporated and product, d-MMTrTpG^{iB}OiB, was precipitated from pyridine (120 ml) by addition to ether (2 l.); weight 9.4 g (88%); silica gel tlc, R_F (20% MeOH–$CHCl_3$) 0.34. The oligomer had the same spectral characteristics as those reported for d-MMTrTpG^{iB} (Caruthers *et al.*, 1972). Removal of the protecting groups from a 54-mg sample gave d-TpG, which had similar R_F values to d-TpG prepared previously by a different route (Ohtsuka *et al.*, 1972). The dimer was completely digested to d-T and d-pG by snake venom phosphodiesterase.

Preparation of d-TpGpG. d-MMTrTpG^{iB}OiB (198 mg, 0.186 mmol) was treated with 1 M NaOH in 4 ml of 50% pyridine–ethanol for 7 min at −5°. The product, d-MMTrTpG^{iB}, obtained after precipitation weighed 165 mg (89%) and had spectral properties similar to those of previously prepared material (Caruthers *et al.*, 1972): silica gel tlc, R_F (20% MeOH–$CHCl_3$) 0.04.

d-MMTrTpG^{iB} (155 mg, 0.156 mmol) and d-pG^{iB}OiB (226 mg, 0.4 mmol) were allowed to react for 4 hr in 1 ml of pyridine containing Me_3PhsCl (175 mg, 0.8 mmol) and then treated overnight with aqueous pyridine. The reaction mixture dissolved in water (14 ml) was extracted with 1-butanol (2 × 15 ml) and the combined butanol extracts were extracted with water (2 × 15 ml) and evaporated. The protecting groups were removed and the residue, after extraction with ether, was dissolved in 100 ml of 10% ethanol and applied to a DEAE-cellulose column in the bicarbonate form. The column was eluted with a linear gradient (0.0–0.25 M, 4 l. total) of triethylammonium bicarbonate in 10% ethanol. Fractions containing d-TpGpG were pooled and the product was isolated by evaporation and lyophilization: weight 112 mg (65%); chromatographic mobility identical with previously prepared d-TpGpG (Ohtsuka *et al.*, 1972). Hydrolysis with snake venom phosphodiesterase gave d-T and d-pG.

Preparation of d-MMTrTp(Et)G^{iB} and d-Tp(Et)G. d-MMTrTpG^{iB}OiBu (8.0 g, 7.52 mmol) was ethylated in *N,N*-dimethylformamide (75 ml), 2,6-lutidine (8.25 ml), and *N*-methylimidazole (6.0 ml) using two (18 ml) charges of ethanol and 2 (14.2 g) charges of TosCl. After work-up, the residue, dissolved in chloroform (750 ml), was extracted with water (750 ml). The product was isolated by silica gel chromatography (5 × 50 cm) using ethyl acetate and 5% methanol in tetrahydrofuran as solvents, followed by precipitation from hexane: weight 3.8 g (51%); silica gel tlc, R_F (10% MeOH–$CHCl_3$) 0.49, trace fluorescent contaminant R_F 0.52. The 3′-*O*-protecting group was removed by treatment with 1 M sodium hydroxide in 60 ml of 50% ethanol–pyridine for 7 min at −5°. After work-up, pure d-MMTrTp(Et)G^{iB} was isolated by silica gel chromatography (3.2 × 50 cm) using 5% methanol–chloroform and 20% methanol–chloroform as solvents: weight 3.0 g (74%); silica gel tlc, R_F (20% MeOH–$CHCl_3$) 0.47.

The protecting groups were removed from a 71-mg sample. The resulting d-Tp(Et)G was purified by paper chro-

matography using solvent I and was isolated by precipitation with ether, weight 24 mg (53%). The triester was resistant to hydrolysis by snake venom and spleen phosphodiesterase.

Preparation of d-MMTrTp(Et)G^{iB}pG^{iB}OiB and d-Tp(Et)GpG. d-MMTrTp(Et)G^{iB} (3.0 g, 3.18 mmol) and d-pG^{iB}OiB (3.3 g, 6.0 mmol) were allowed to react for 4 hr in pyridine (15 ml) containing Me_3PhsCl (2.63 g, 12 mmol). Following aqueous pyridine treatment, the aqueous solution (210 ml) was extracted with 1-butanol (2 × 240 ml). The combined butanol extracts were extracted with water (2 × 250 ml) and evaporated, and the residue was chromatographed on a silica gel column (3.5 × 50 cm). The column was eluted with 10% methanol in chloroform (2 l.) followed by 20% methanol in chloroform (3 l.). Fractions were obtained which contained the desired d-MMTrTp(Et)G^{iB}pG^{iB}OiB as well as some detritylated material, d-Tp(Et)G^{iB}pG^{iB}OiB. The total material from these fractions was treated with 428 mg (1.3 mmol) of monomethoxytrityl chloride in pyridine (3 ml) for 21 hr followed by 95% ethanol (2 ml) for 2 hr. The solvents were evaporated and the desired d-MMTrTp(Et)G^{iB}pG^{iB}OiB was isolated by precipitation with ether; weight 2.39 g (50%); silica gel tlc, R_F (20% MeOH-$CHCl_3$) 0.13; R_F (30% MeOH-$CHCl_3$) 0.47.

The protecting groups were removed from a 25-mg sample and the resulting d-Tp(Et)GpG was isolated after paper chromatography using solvent F. Incubation with snake venom phosphodiesterase (16 hr, 37°) gave d-Tp(Et)GpG, dpG, and d-Tp(Et)G. The oligomer was resistant to hydrolysis by spleen phosphodiesterase.

Preparation of d-MMTrTp(Et)G^{iB}p(Et)G^{iB}OiB and d-Tp(Et)Gp(Et)G. d-MMTrTp(Et)G^{iB}pG^{iB}OiB (580 mg, 0.39 mmol) was ethylated in *N,N*-dimethylformamide (8 ml) and 2,6-lutidine (4 ml) using 2 (4 ml) charges of ethanol and 2 (1.55 g, 8 mmol) charges of TosCl. After work-up, a chloroform (40 ml) solution of the residue was extracted with water (2 × 40 ml). The triester was purified by silica gel chromatography (2 × 30 cm) using chloroform (400 ml) and 10% methanol-chloroform (400 ml) as solvents. The resulting d-MMTrTp(Et)G^{iB}p(Et)G^{iB}OiB weighed 176 mg (31%); silica gel tlc, R_F (10% MeOH-$CHCl_3$) 0.29.

The protecting groups were removed from a 160-mg sample and the resulting d-Tp(Et)Gp(Et)G was purified by paper chromatography using solvent I; weight after precipitation with ether, 35 mg (33%). The triester was resistant to hydrolysis by snake venom phosphodiesterase.

Preparation of d-GpGpT. d-MMTrG^{iB}pG^{iB} (0.2 mmol; Agarwal *et al.*, 1972) was allowed to react with d-pTOAc (177 mg, 0.4 mmol) for 2.5 hr in pyridine (1 ml) containing Me_3PhsCl (175 mg, 0.8 mmol), followed by overnight aqueous pyridine treatment. The reaction mixture in 14 ml of water was extracted with 2 (15 ml) portions of 1-butanol, and the residue obtained after evaporation of the butanol was treated to remove protecting groups. Following extraction with ether, the residue was applied to a DEAE-cellulose column in the bicarbonate form and the column was eluted with a linear gradient of triethylammonium bicarbonate (0.0–0.50 M, 4 l. total). Fractions containing the desired d-GpGpT were pooled, evaporated, and lyophilized to give 14 mg (6%) of oligomer. Hydrolysis with snake venom phosphodiesterase gave d-G, dpG, and d-pT.

Preparation of d-MMTrTp(Et)TOAc. d-MMTrTpTOAc (3.58 g, 4.0 mmol, prepared by condensation of d-MMTrT with d-pTOAc) was ethylated in *N,N*-dimethylformamide (70 ml)–2,6-lutidine (35 ml) with 2 (35 ml) charges of ethanol and 2 (13.3 g, 70 mmol) charges of TosCl. Following work-up, a 200-ml chloroform solution of the residue was extracted with water (3 × 200 ml). The product was isolated by silica gel column chromatography (4 × 30 cm) using ether and tetrahydrofuran as solvents. The resulting d-MMTrTp(Et)TOAc was precipitated with hexane: weight 1.71 g (50%); silica gel tlc, R_F (EtOAc) 0.18; R_F (THF) 0.59. The material was identical with material previously prepared by a different synthetic route (DeBoer *et al.*, 1973). Removal of the protecting groups gave d-Tp(Et)T identical with an authentic sample (Miller *et al.*, 1971).

Preparation of d-MMTrTp(Et)TpC^{An}OAc and d-Tp(Et)TpC. d-MMTrTp(Et)T (1.89 mmol), prepared by hydrolysis of d-MMTrTp(Et)TOAc (1.71 g, 1.89 mmol) with 1 M NaOH in 10 ml of 50% pyridine, was allowed to react with d-pC^{An}OAc (1.13 g, 2.0 mmol) for 3 hr in 10 ml of pyridine containing Me_3PhsCl (880 mg, 4 mmol). Following aqueous pyridine treatment, the reaction mixture in 100 ml of water was extracted with 1-butanol (2 × 100 ml). The residue obtained after evaporation of the butanol was chromatographed on a silica gel column (2 × 40 cm) using 500 ml of ethyl acetate, 1 l. of tetrahydrofuran, and 20% methanol in chloroform, which eluted the product. The resulting d-MMTrTp(Et)TpC^{An}OAc, which was precipitated from pyridine by addition to ether, weighed 1.33 g (51%); silica gel tlc, R_F (THF) 0.07, R_F (20% MeOH-$CHCl_3$) 0.42. A sample of the material was treated to remove protecting groups and the resulting d-Tp(Et)TpC was purified by paper chromatography using solvent I. Hydrolysis with snake venom phosphodiesterase gave d-Tp(Et)T and d-pC. The trimer was resistant to hydrolysis by spleen phosphodiesterase.

Preparation of d-MMTrTp(Et)Tp(Et)C^{An}OAc and d-Tp(Et)Tp(Et)C. d-MMTrTp(Et)TpC^{An}OAc (1.23 g, 0.9 mmol) was ethylated in a solution of *N,N*-dimethylformamide (18 ml)–2,6-lutidine (9 ml) using 2 (9 ml) charges of ethanol and 2 (3.4 g, 18 mmol) charges of TosCl. After work-up, the residue in chloroform (50 ml) was extracted with water (2 × 50 ml) and dried over anhydrous sodium sulfate. Column chromatography on silica gel (2 × 35 cm) using ether (350 ml), tetrahydrofuran (500 ml), and 20% methanol in chloroform (150 ml) gave, after precipitation with hexane, d-MMTrTp(Et)Tp(Et)C^{An}OAc: 3.93 g (32%); silica gel tlc, R_F (THF) 0.45, R_F (20% MeOH-$CHCl_3$) 0.70.

The protecting groups were removed from a 20-mg sample and the resulting d-Tp(Et)Tp(Et)C was purified by paper chromatography using solvent I. The triester was resistant to hydrolysis by snake venom and spleen phosphodiesterase.

Preparation of d-MMTrTp(Et)Tp(Et)C^{An}pA^{Bz}OAc and d-Tp(Et)Tp(Et)CpA. The 3′-acetyl group was removed from d-MMTrTp(Et)Tp(Et)C^{An}OAc (366 mg, 0.27 mmol) by hydrolysis with 1 M NaOH in 50% pyridine for 15 min. Following work-up and isolation by precipitation from hexane, 350 mg (97%) of d-MMTrTp(Et)Tp(Et)C^{An} was obtained; silica gel tlc, R_F (THF) 0.33; R_F (20% MeOH-$CHCl_3$) 0.42. The triester was condensed with d-pA^{Bz}OAc (166 mg, 0.3 mmol) for 3.5 hr in pyridine (2 ml) containing Me_3PhsCl (132 mg, 0.6 mmol). Following aqueous pyridine treatment, the solution was diluted with water (50 ml) and the product was extracted into 1-butanol (3 × 50 ml). Col-

umn chromatography on silica gel (2 × 21 cm) using ether (150 ml), tetrahydrofuran (400 ml), and 20% methanol-chloroform (200 ml) gave d-MMTrTp(Et)Tp(Et)C^{An}p-A^{Bz}OAc (295 mg, 60%) after precipitation with hexane; silica gel tlc, R_F (THF) 0.03; R_F (20% MeOH-CHCl$_3$) 0.40.

The protecting groups were removed from a 10-mg sample and the resulting d-Tp(Et)Tp(Et)CpA was purified by paper chromatography using solvent I. Hydrolysis with snake venom phosphodiesterase gave d-Tp(Et)Tp(Et)C and d-pA. The tetramer was resistant to hydrolysis by spleen phosphodiesterase.

Preparation of d-MMTrTp(Et)Tp(Et)C^{An}p(Et)A^{An}OAc and d-Tp(Et)Tp(Et)Cp(Et)A. d-MMTrTp(Et)Tp(Et)C^{An}p-A^{Bz}OAc (285 mg, 0.155 mmol) was ethylated in a solution containing *N,N*-dimethylformamide (5 ml)–2,6-lutidine (2.5 ml) with 2 (2.5 ml) charges of ethanol and 2 (0.95 g, 5 mmol) charges of TosCl. After work-up, the residue in chloroform (100 ml) was extracted with water (2 × 100 ml) and chromatographed on a silica gel column (2 × 30 cm) using ethyl acetate (300 ml), tetrahydrofuran (700 ml), and 20% methanol-chloroform (200 ml). d-MMTrTp(Et)Tp-(Et)C^{An}p(Et)A^{Bz}OAc was isolated by precipitation into hexane: weight 182 mg (63%); silica gel tlc, R_F (THF) 0.39, R_F (20% MeOH-CHCl$_3$) 0.73.

The protecting groups were removed and the resulting d-Tp(Et)Tp(Et)Cp(Et)A was purified by paper chromatography using solvent I. The tetramer was completely resistant to hydrolysis by snake venom and spleen phosphodiesterase.

Preparation of d-TpTpCpA. d-DMTrTpT (100 mg, 0.1 mmol; Narang and Khorana, 1965) and d-pC^{Bz}pA^{Bz}OAc (55 mg, 0.25 mmol; prepared in a manner similar to that used for the preparation of d-pC^{An}pA^{Bz}OAc; Weber and Khorana, 1972) were condensed for 4 hr in 3 ml of pyridine containing Me$_3$PhsCl (66 mg, 0.3 mmol). After aqueous pyridine treatment the protecting groups were removed and the reaction mixture was chromatographed on a DEAE-cellulose column (2.5 × 40 cm) in the bicarbonate form using a linear gradient of triethylammonium bicarbonate (0.05–0.30 M, 4 l. total). Tetramer which was contaminated with a small amount of d-pCpA was isolated and incubated with 0.45 mg of alkaline phosphatase in 1 ml of 150 mM Tris (pH 8.2) for 4 hr at 37°. The digest, after extraction with chloroform–isoamyl alcohol (5:2, v/v), was chromatographed on a second DEAE-cellulose column (1.2 × 30 cm) using a linear triethylammonium bicarbonate gradient (0.0–0.25 M, 2 l. total). The desired tetramer, d-TpTpCpA (750 A_{260} units, 37%), was isolated and lyophilized. The oligomer was completely digested with snake venom phosphodiesterase to give d-T, d-pT, d-pC, and d-pA, and with spleen phosphodiesterase to give d-Tp, d-Cp, and d-A.

Equilibrium Dialysis Experiments

^{3}H-Labeled Oligodeoxyribonucleotides and Oligodeoxyribonucleotide Triesters. One to four milligrams of each of the oligomers was heated at 90° in a sealed tube with 100 µl of tritiated water (2.5 Ci/mmol). The adenine-containing oligomers were heated for 3 hr while the guanine-containing oligomers were heated for 5.5 hr. The oligomers were purified by paper chromatography using solvents C and I, after lyophilization from water several times to remove exchangeable tritium. The results of the exchange reactions are presented in Table III.

^{3}H-Labeled Ribooligonucleotides. ^{3}H-Labeled ribooligonucleotides (sp act. 0.35–3.2 Ci/mmol) were prepared using primer dependent polynucleotide phosphorylase as described by Uhlenbeck *et al.* (1970). After purification by paper chromatography, the oligomers were characterized by digestion with RNase T$_1$ or RNase A.

Purified tRNAs. tRNA$^{Phe}{}_{coli}$ was prepared by a three-step procedure: (1) fractionation of crude tRNA$_{coli}$ on a BD-cellulose column (Roy and Söll, 1968); (2) acylation of the resulting tRNA^{Phe} fraction and purification of Phe-tRNA^{Phe} on BD-cellulose (Gillam and Tener, 1971); (3) deacylation followed by reversed-phase chromatography of the tRNA^{Phe} on RPC 5 (Pearson *et al.*, 1971). The resulting tRNA^{Phe} had an amino acid acceptance activity of 1700 pmol of phenylalanine/A_{260} unit and was considered greater than 90% pure.

tRNA$^{Phe}{}_{yeast}$ which accepted 1600 pmol of phenylalanine/A_{260} unit was a generous gift from Professor Friederich Cramer and was considered greater than 90% pure. The extinction coefficients used were: tRNA$^{Phe}{}_{coli}$ ϵ_{258} 5.43 × 10^5, tRNA$^{Phe}{}_{yeast}$ ϵ_{258} 5.03 × 10^5 (Blum, 1971).

The Y base was removed from tRNA$^{Phe}{}_{yeast}$ using the procedure of Thiebe and Zachau (1970). After reaction, the reaction mixture was applied to a BD-cellulose column. The column was first eluted with 1.2 M NaCl–50 mM NaOAc (pH 5.0)–10 mM MgCl$_2$ which eluted tRNA^{Phe} minus Y base, and then with 1.2 M NaCl–50 mM NaOAc (pH 5.0)–10 mM MgCl$_2$ containing 15% ethanol, which eluted a small amount of unreacted tRNA$^{Phe}{}_{yeast}$. The 3′-CA terminus was removed from tRNA$^{Phe}{}_{yeast}$ following the method of Simsek *et al.* (1973). The reaction mixture was applied to a DEAE-cellulose column. The column was eluted with 0.3 M NaCl–50 mM Tris-HCl (pH 8.0) which removed the snake venom phosphodiesterase and mononucleotides. The column was further eluted with 1 M NaCl–50 mM Tris-HCl (pH 8.0) which removed the tRNA.

Dialysis Experiments. Dialysis experiments were carried out in 30- and 50-µl Plexiglass chambers separated by a dialysis membrane using the methods described by Uhlenbeck (1972). Precautions were taken to prevent contamination by ribonuclease. tRNA solutions (40 µM) were introduced into side A and oligomer solutions (0.1–46 µM, final concentration) into side B. After equilibration at 0° (from 4 to 14 days), two (10 µl) samples from each side were each diluted with 100 µl of water and counted in 10 ml of 4% Biosolv-toluene cocktail. For experiments where the oligomer concentration was greater than 0.5 µM, K was calculated using the equation: $K = (R - 1)/(\text{tRNA} - [\text{O}]b)$, where $[\text{O}]b = (\text{cpm A} - \text{cpm B})/(\text{cpm A} + \text{cpm B})[\text{O}]$ and $[\text{O}]$ = the initial oligomer concentration (Högenauer, 1970). To ensure attainment of equilibrium, experiments were checked on successive days until no further change in K was observed.

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A Polyribonucleotide Containing Alternating $\rightarrow P = O$ and $\rightarrow P = S$ Linkages

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The enzymatic synthesis of the polyribonucleotide $\mathrm{A}\overset{\mathrm{S}}{\mathrm{p}}\mathrm{Up}(\mathrm{A}\overset{\mathrm{S}}{\mathrm{p}}\mathrm{Up})_n$ by the use of RNA polymerase is described. Snake venom and spleen phosphodiesterase degrade this polymer at a very slow rate compared to poly r(A—U). The dinucleotide resulting from digestion with pancreatic ribonuclease is resistant to spleen phosphodiesterase.

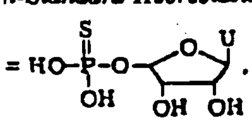
Marked changes in some properties of nucleotides are brought about by the replacement of the $\rightarrow P = O$ group by a $\rightarrow P = S$ group. Nucleoside 5′-phosphorothioates, for example, are resistant to a number of phosphatases [1] and dinucleoside phosphorothioates are resistant to some diesterase [2, 3].

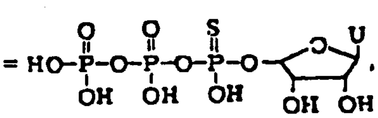
It is of interest therefore, to ascertain whether polymers which contain thiophosphate linkages are also resistant to these diesterases. The synthesis of such polymers has been attempted enzymatically with DNA dependant RNA-polymerase.

MATERIALS AND METHODS

UTPS was synthesized as described previously [4]; [^{14}C]ATP (specific activity 0.25 mC/mmole) and [^{14}C]UTP (specific activity 0.575 mC/mmole) were purchased from Schwarz Bioresearch, Inc. (Orangeburg, N. Y., U.S.A.). ATP, phosphoenolpyruvate and pyruvate kinase were purchased from Boehringer & Soehne (Mannheim, Germany); UTP was purchased from PL Biochemicals, Inc. (Milwaukee, Wis., U.S.A.).

Non-Standard Abbreviations.

UMPS =  , uridine-5′-phosphorothioate;

UTPS =  , uridine-5′-triphosphorothioate;

poly r(A·US) = Copolymer of AMP and UMPS.

Enzymes. DNA-dependent RNA polymerase or nucleoside triphosphate: RNA nucleotidyltransferase (E.C. 2.7.7.6); spleen phosphodiesterase (E.C. 3.1.4.1); snake venom phosphodiesterase (E.C. 3.1.4.1); spleen phosphomonoesterase or orthophosphoric monoester phosphohydrolase (E.C. 3.1.3.2); DNA polymerase or deoxynucleosidetriphosphate: DNA deoxynucleotidyltransferase (E.C. 2.7.7.7); pancreatic ribonuclease (E.C. 2.7.7.16).

DNA-dependent RNA-polymerase was purified according to the method of W. Zillig *et al.* [5] up to the sucrose gradient step. Its specific activity was, in the definition of Zillig, 450 E.U./mg protein (determination of protein according to Lowry *et al.* [6]).

Spleen phosphodiesterase and spleen phosphomonoesterase were generously donated by Dr. H. Sternbach, Göttingen. Snake venom phosphodiesterase and pancreatic ribonuclease were purchased from Boehringer & Soehne (Mannheim, Germany). Poly d(A—T) was generously supplied by Dr. A. Lezius, Göttingen. Paper chromatography was carried out by the descending method in system A: 2-propanol—conc. ammonia—water (6:3:1, v/v/v) on paper Schleicher and Schüll 2043 b, washed.

Electrophoresis was performed on the same paper with buffer: 0.05 M ammonium formate (pH 3.5).

Melting curves were taken with a Gilford Mod. 2000 recorder connected with a Beckman Mod. DUR spectrophotometer in combination with a Neslab LTP-1 temperature programmer.

Polymerization

The incubation mixture for the polymerization with RNA-polymerase contained, unless otherwise stated, 0.03 M Tris-acetate (pH 7.9), 0.03 M magnesium acetate, 0.13 M NH_4Cl, 1 mM each of [^{14}C]ATP (specific activity 0.25 mC/mmole) and UTP or UTPS, 0.02 M phosphoenolpyruvate (Na-salt), 20 µg/ml pyruvate kinase, poly d(A—T) and RNA-polymerase as indicated. Incubation temperature was 37°.

To follow the progress of polymerization, aliquots (0.010 or 0.025 ml) were taken after certain time intervals and applied to paper strips (2×10 cm^2) (DEAE-cellulose Whatman DE 81 [7]). After eluting with 0.3 M ammonium formate and drying, the polymeric material remaining at the starting zone was cut out and counted in a liquid scintillation counter (Tricarb, Mod. 4312).

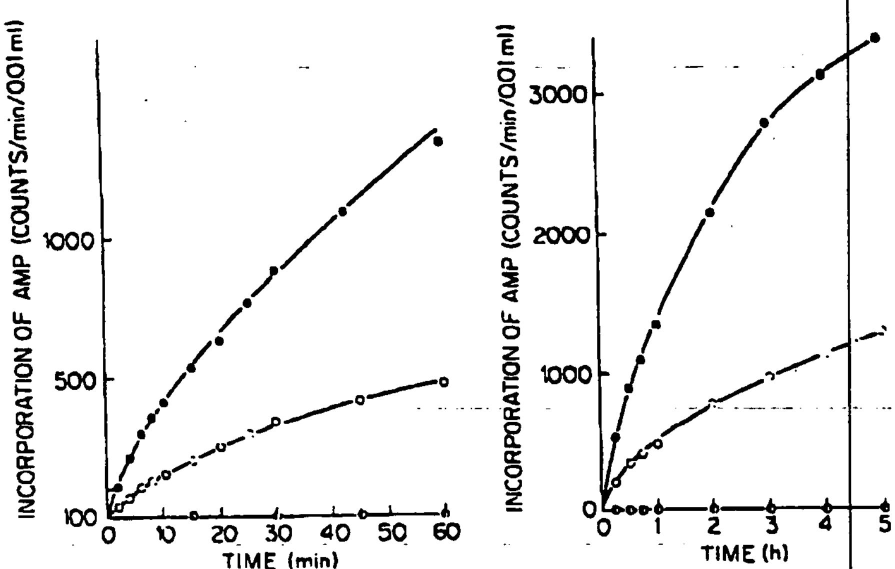


Fig. 1. *Incorporation of AMP and UMP (●) or AMP and UMPS (○) with RNA polymerase on poly d (A—T) template.* Control: Incorporation of AMP in absence of UTP (◐). In addition to the compounds mentioned under Materials and Methods, the incubation mixtures (1.0 ml) contained: RNA polymerase, 44 µg of protein; poly d (A—T), 1.0 absorbance unit at 260 mµ

Isolation of Product

After polymerization for about 5—6 h, 0.3 ml of 1 M NaCl solution was added to 0.3 ml of the incubation mixture and the polymer precipitated with 3 ml of ethanol at 0°. The precipitate was centrifuged for 10 min at 10,000 rev./min (Servall SS 34). The pellet was dissolved in 2.0 ml of water and dialyzed against 0.1 M NaCl/0.1 M Tris-acetate (pH 8.9) for 4—5 h at 2°; this was followed by a second dialysis, over-night, at 2° against the buffer used for the particular enzymatic degradation.

Conditions for the enzymatic degradations are given under the appropriate figures.

RESULTS

POLYMERIZATION EXPERIMENTS

Fig. 1 shows the incorporation of [^{14}C]ATP into the polymer as a function of time in the presence of UTP or UTPS, using poly d (A—T) as a template.

From Fig. 1 it was clear that UTPS could serve as substrate for DNA dependent RNA polymerase instead of UTP. However, the polymer was formed at a slower rate. In order to get a quantitative relationship between the two substrates UTP and UTPS, polymerization was carried out with different concentrations of equimolar mixtures of [^{14}C]ATP and UTP as well as [^{14}C]ATP and UTPS. Fig. 2 shows the rate of polymerization as a function of substrate concentration. Fig. 3 represents the Lineweaver-Burk-plot.

To determine the ratio of incorporated [^{14}C]-AMP/UMPS in the modified polymer, the isolated polymer was dissolved in 2 ml of buffer (containing 50 mM Tris-HCl, pH 8.1; 0.5 mM $MgCl_2$ and 10.0 mM orthophosphate, Na^+-salt) and subjected to alkaline hydrolysis. On addition of 0.86 ml of 1 M KOH, the

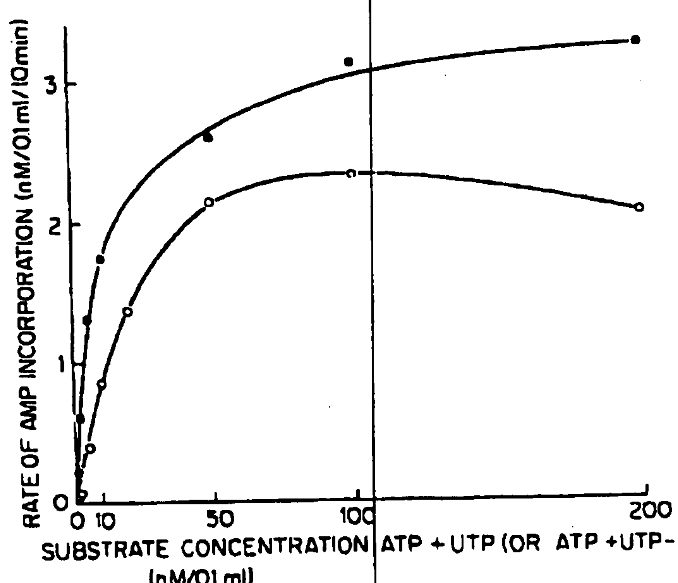


Fig. 2. *Rate of incorporation of AMP and UMP (●) or AMP and UMPS (○) in presence of increasing amounts of substrates.* In addition to the compounds mentioned under Materials and Methods 0.1 ml of incubation mixture contained: RNA polymerase, 44 µg of protein; poly d(A—T), 0.05 absorbance units at 260 mµ; increasing amounts of substrates. After incubation for 10 min at 37°, the reaction mixture was cooled in an ice bath and the reaction stopped by addition of 0.2 ml of an aqueous solution of 1 mg/ml of bovine serum albumin and 0.3 ml of 10% trichloroacetic acid (w/v). The acid insoluble material was filtered on membrane filters (MF 50, Sartorius Membranfiltergesellschaft, Göttingen) and repeatedly washed with ice cold 5% trichloroacetic acid. After drying the radioactivity was counted in a liquid scintillation counter

absorbance of this solution at 260 mμ increased from 0.75 to 0.98 (= 2.80 absorbance units) during 18 h at 37°.

The radioactivity of 0.02 ml of this solution was measured, and yielded 285.4 counts/min = 40,700 counts/min for 2.86 ml. This is equal to 0.125 μmoles of incorporated [14C]AMP (Standard: 32,500 counts/min/0.100 μmoles [14C]ATP); this is equal to 1.92 absorbance units at 260 mμ. To obtain the amount

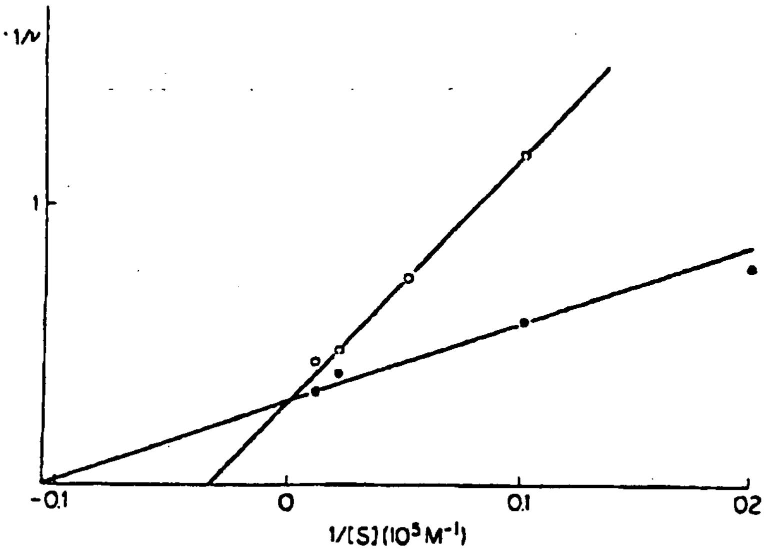


Fig. 3. *Lineweaver-Burk plot of the rate of incorporation of AMP and UMP (●) or AMP and UMPS (○)*

of incorporated UMPS one has to substract the amount of incorporated [14C]AMP (1.92 absorbance units at 260 mμ) from the total number of absorbance units (2.80). The difference (0.88 absorbance units) is equal to 0.113 μmoles of UMPS if $\varepsilon = 7{,}800$ at pH 11. This shows that [14C]AMP and UMPS were incorporated approximately to the same extent.

Thermal Melting

Thermal melting profiles of poly r(A—U) and poly r(A—US) are shown in Fig. 4. The transition at 55° represents the melting of the template poly d(A—T).

DEGRADATION EXPERIMENTS

Degradation with Pancreatic Ribonuclease

To degrade the polymer with pancreatic ribonuclease, 2 μg of protein was added to 0.100 ml of polymerization mixture and kept at 25°. To follow the degradation of the polymer 0.01 ml were withdrawn after certain time intervals, applied to DEAE paper as described under Materials and Methods, eluted with 5% trichloroacetic acid (w/v) at 2° and checked for undegraded polymer in the scintillation counter.

Fig. 5 shows the degradation of the unmodified and the modified polymers by pancreatic ribonuclease as a function of time at different temperatures.

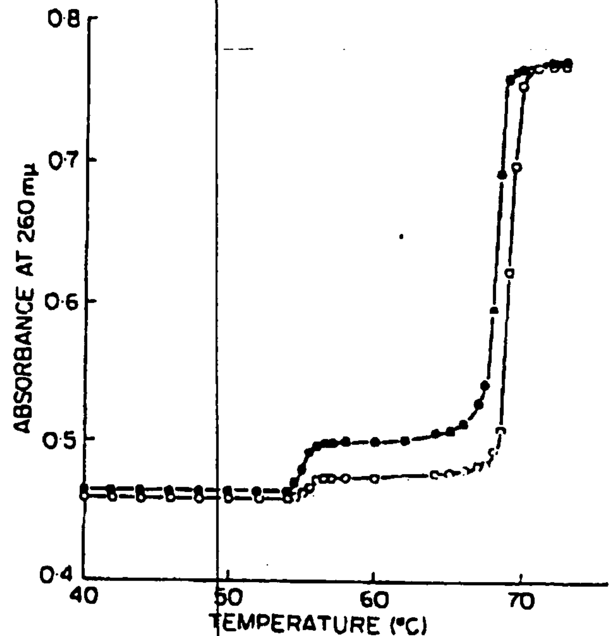


Fig. 4. *Temperature profiles of poly r(A—U) and poly r(A—US).* Isolated polymer was dialyzed against 0.01 M Tris HCl (pH 8.1), 0.01 M Na_2HPO_4, 0.5 mM $MgCl_2$. Increase of ultraviolet absorbance is plotted *versus* temperature. ○, poly r(A—U); ●, poly r(A—US)

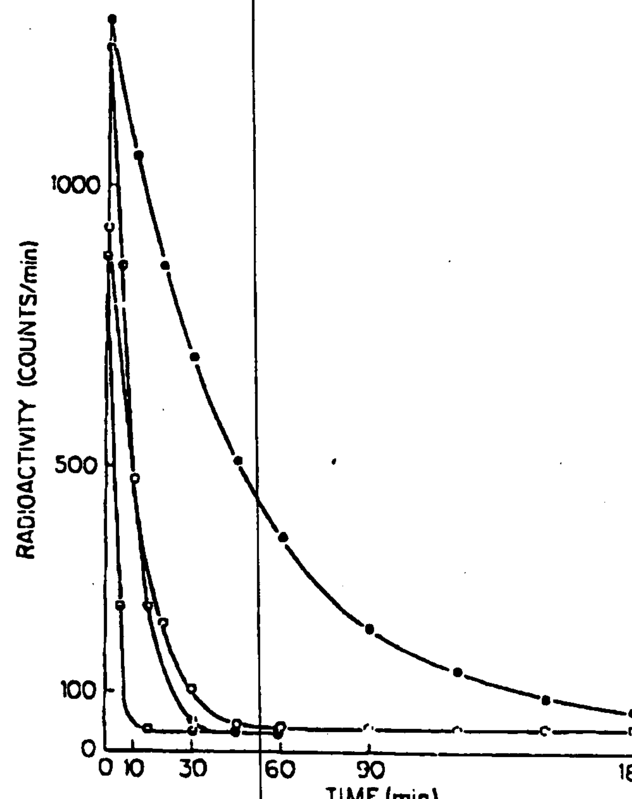


Fig. 5. *Degradation of the unmodified and the modified polymer by pancreatic ribonuclease as a function of time.* ●, degradation of poly r(A—U) at 25°; ○, degradation of poly r(A—US) at 25°; ⊖, degradation of poly r(A—U) at 37°; ◐, degradation of poly r(A—US) at 37°

The two polymers show no marked difference in degradation velocity considering that the relative amount of enzyme for the modified polymer was

approximately 35 % higher than for the natural polymer.

For the isolation of the expected dinucleotides, separate experiments were run at 37°. The incubation mixture was then chromatographed on paper in System A.

The ultraviolet-active material was cut out, eluted and subjected to electrophoresis (pH 3.5). Apart from some unreacted triphosphates there was one ultraviolet-active spot (electrophoretic mobility 9.5 cm). This material (7 absorbance units at 260 mμ) was incubated with spleen phosphodiesterase at pH 6.0 for 3.5 h at 37°. After separation by paper-chromatography in System A, the ultraviolet-active material was cut out, eluted and subjected to electrophoresis (pH 3.5). On degradation of the unmodified polymer, electrophoresis yielded 2 spots which were identical with authentic AMP and UMP (electrophoretic mobility 6.5 cm and 13.0 cm), while in the case of the modified polymer electrophoresis yielded only 1 spot (electrophoretic mobility 9.5 cm). One expects the latter for a dinucleoside diphosphate of the type $\overset{\overset{S}{\|}}{\mathrm{ApUp}}$ [3].

Taking into account the results of the alkaline hydrolysis, formation of this stable dinucleoside diphosphate suggests the following structure for the modified polymer:

$$\mathrm{pppA}\overset{\overset{S}{\|}}{\mathrm{p}}\mathrm{U}\overset{\overset{S}{\|}}{\mathrm{p}}(\mathrm{ApUp})_n$$

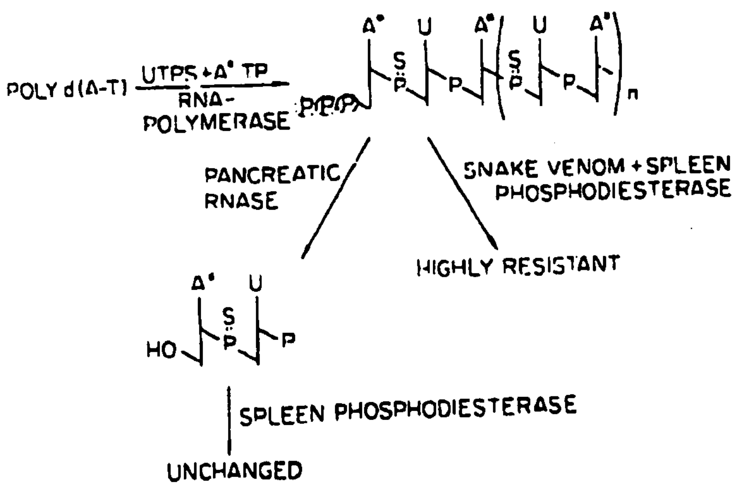


Fig. 6

Degradation with Snake Venom and Spleen Phosphodiesterase

The isolated polymer was dialyzed against a solution of 0.1 M NaCl and 0.1 M Tris acetate (pH 8.9). Fig. 7 shows the degradation of the modified and unmodified polymers by snake venom phosphodiesterase as a function of time. One can see clearly that the

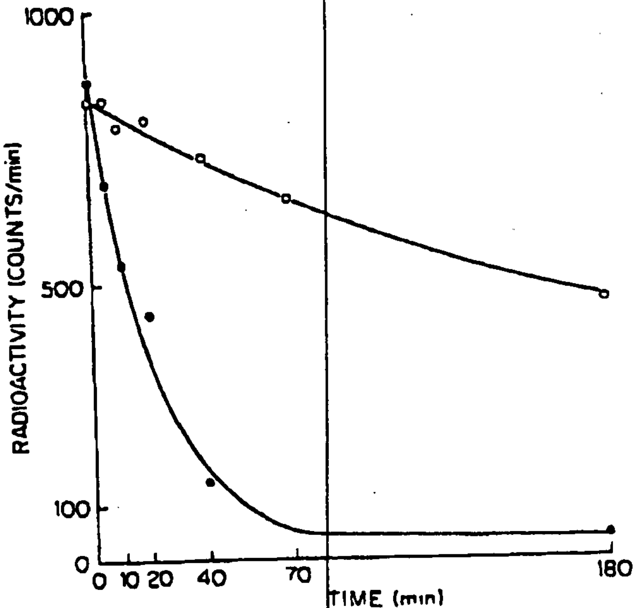


Fig. 7. *Degradation of the unmodified (●) and the modified (○) polymer by snake venom phosphodiesterase as a function of time.* 0.45 ml of buffer contained 0.24 absorbance units of the unmodified or modified polymer. 0.05 ml were taken for time 0 value and the reaction solution kept at 37° for 10 min; 10 μg of enzyme were then added, aliquots taken at certain intervals, applied on DEAE-paper, eluted with 0.3 M ammonium formate at 2° and the radioactivity counted as described under Materials and Methods

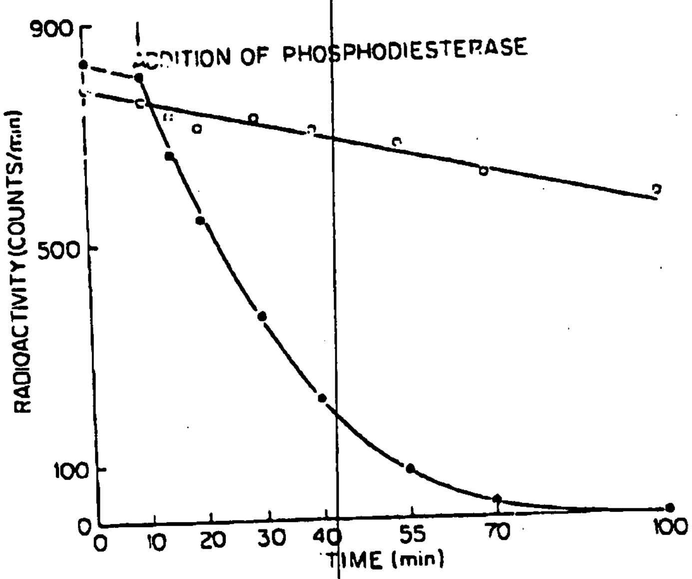


Fig. 8. *Degradation of the unmodified (●) and the modified (○) polymer by phosphomonoesterase and phosphodiesterase from spleen.* To 0.45 ml of the same buffer solution of polymer as described for the degradation by snake venom was added 0.005 ml of 0.1 M EDTA. The solution was then brought to pH 5.25 by conc. HCl. For time 0 value 0.05 ml were with-drawn, the reaction solution kept at 37° for 5 min and then 0.15 μg of phosphomonoesterase was added. After 10 min 0.05 ml were withdrawn and to the remaining solution was added 7.5 μg of spleen phosphodiesterase. Aliquots were taken, applied on DEAE paper, eluted and checked for undegraded material as described for the degradation with pancreatic ribonuclease

modified polymer is degraded by snake venom phosphodiesterase at a much slower rate than the unmodified polymer.

For the degradation of the polymers with spleen phosphodiesterase it was necessary to remove the terminal 5'-phosphate of the polymer. This was done by preincubating the solutions of the modified and the unmodified polymers for a short period with spleen phosphomonoesterase and then adding diesterase. Fig. 8 shows the degradation of the polymers with these two enzymes.

DISCUSSION

Because of the ambident character of nucleoside phosphorothioates the chemical synthesis of polymers which contain → P = S linkages is not practical. The possibility of enzymatic synthesis was therefore investigated after we had obtained the necessary modified triphosphates [4]. To the best of our knowledge no attempts to polymerize nucleoside triphosphates modified at the α-P atom *e.g.* nucleoside phosphonopyrophosphates have been successful [8, 9]. It was therefore an open question whether such nucleoside 5'-polyphosphorothioates would be accepted by polymerizing enzymes such as DNA-dependent DNA- and RNA-polymerase and polynucleotide phosphorylase. Although we have tested these three enzymes with the necessary nucleoside polyphosphate analogs as substrates [10] we have only been successful so far in obtaining polymers with → P = S linkage with DNA-dependent RNA-polymerase using UTPS as modified substrate. Because poly d(A—T) was our template the second substrate was ATP. The polymer therefore was expected to contain alternating → P = O, → P = S groups.

When using UTPS instead of UTP in the polymerization with RNA-polymerase, the initial velocity of polymerization as well as the degree of polymerization is $^1/_3$ as great (Fig. 1). As can be seen from the Lineweaver-Burk plot, $K_m = 3 \times 10^{-4}$ M for the modified substrate, whereas $K_m = 9.5 \times 10^{-5}$ M for the unmodified substrate. If one assumes K_m to represent the real dissociation constant of the enzyme-substrate complex in the presence of template then the affinity of the enzyme for the thiophosphate is only about $^1/_3$ as great as the affinity for the normal substrate. This decrease of affinity seems to be the reason for the slower initial velocity. This is indicated in Fig. 3 by an increase in the values of K_m while V_{max} remains constant.

A comparison of the melting profiles (Fig. 4) of poly r(A—U) and poly r(A—US) shows that the introduction of P = S linkages does not interfere with the formation of a double strand. The difference in T_m for the two polymers was found to be about 1°. Further investigation will be required to show if this difference is of any significance.

One expects a polymer like poly r(A—US) to be resistant to exonucleases but to be degraded by endonucleases. According to our expectations we found it to be degraded by pancreatic ribonuclease.

As one can see from Fig. 7 and 8, an incubation with spleen as well as snake venom phosphodiesterase, poly r(A—U) is completely degraded within about one hour whereas poly r(A—US) is only 15—20% degraded. This slow degradation of poly r(A—US) could be due either to some endonucleolytic activity present in the enzyme preparations or to some sensitivity of the → P = S linkages to these enzymes. This question may be resolved by investigating the degradation of polymers with → P = S linkages exclusively.

The resistance of poly r(A—US) to snake venom phosphodiesterase is in contrast to the apparently facile degradation of poly-5'-thiouridylic acid crude rattlesnake venom [11]. This difference in behaviour by O.S- and O.O-substituted diesters of phosphorothioic acid is repeated on the monoester level. There, S-substituted esters are cleaved by alkaline phosphatase [12] whereas O-substituted esters are not.

From the results of these investigations we feel that polymers containing exclusively → P = S linkages may lend themselves well to the study of interactions of polyribonucleotides with ribosomes and might be useful as messenger RNA.

The authors wish to thank Prof. Dr. F. Cramer for helpful discussions and generous support of this work, Mrs. G. Stelzer and Mr. R. Rackwitz for skilful technical assistance.

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Synthesis and enzymatic properties of deoxyribooligonucleotides containing methyl and phenylphosphonate linkages

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ABSTRACT

Chemical methods for the synthesis of short deoxyribooligonucleotides containing methyl and phenylphosphonodiester linkages have been developed. The interaction of two such nonionic dinucleotide analogs, $T(pCH_3)T$ and $T(pC_6H_5)T$, with several enzymes has been investigated. Because of the phosphonate linkage each dinucleotide exists as a diastereomeric pair as shown by thin layer chromatography and enzymatic studies. Both isomers of each dinucleotide can be phosphorylated by T_4-polynucleotide kinase in the presence of [γ-^{32}P]ATP. Only one of the diastereoisomers of each dinucleotide is slowly hydrolyzed by snake venom phosphodiesterase and acts as an inhibitor of the enzyme-catalyzed hydrolysis of 5'-labeled oligothymidylic acid. Both isomers of each dinucleotide analog are completely resistant to hydrolysis by spleen phosphodiesterase.

INTRODUCTION

A great number of nucleotide analogs in which either the heterocyclic base or the sugar is modified have been synthesized and studied while only a small number of analogs are known in which the phosphate group is altered. These include nucleoside phosphites[1], nucleoside phosphonates[2], and nucleoside phosphorothioates[3]. Oligonucleotides containing altered phosphate groups can be classified into three major groups: (a) polyionic oligonucleotides, (b) nonionic oligonucleotides, and (c) mixed ionic-nonionic oligonucleotides. Oligonucleotides with a phosphorothioate group in place of a phosphate group were synthesized by Eckstein and have proved to be useful substrates in studying enzyme mechanisms[4]. Nonionic analogs such as oligonucleotide triesters have been synthesized by Ts'o and coworkers[5] who used them as model compounds for studies on the physical chemistry of nucleic acids. More recently these authors have obtained deoxyribodinucleoside methylphosphonates and studied their physical properties by CD and pmr spectroscopy[6]. However, chemical synthesis of these nonionic analogs has not been reported by these authors.

Independently, we have developed chemical procedures for the synthesis of oligonucleotides containing nonionic as well as mixed diester linkages with the hope of acquiring potential pseudosubstrates for the nucleic acid enzymes. In this paper we wish to report the synthesis of oligonucleotides carrying methyl and phenylphosphonate linkages, their phosphorylation by T_4-polynucleotide kinase, and their interaction with snake venom and spleen phosphodiesterases.

RESULTS AND DISCUSSION

Although several procedures for the synthesis of dinucleoside methyl and phenylphosphonates were attempted, only three procedures were found satisfactory and are described below. Methods A and B produced dinucleoside methylphosphonates, but in each case the reaction was slow and the yield of final product was low. In Method A methylphosphonodichloridate was used directly for phosphorylation of the 3'-hydroxyl group of a 5'- and amino-protected nucleoside. The 3'-phosphorylated nucleoside thus obtained was further treated with the 5'-hydroxyl group of incoming nucleoside. While the first step of phosphorylation was quantitative in 2 hr, the second step of phosphorylation was slow, requiring 12 hr for the formation of diester in 40% yield. Although dinucleoside methylphosphonates, d(Me$_2$O)TrT(pCH$_3$)T and d(Me$_2$O)Tr anC (pCH$_3$)anC[7], were isolated in reasonable yields (36% and 28%, respectively) using Method A, the dinucleotide, d(Me$_2$O)Tr ibG(pCH$_3$)ibG was isolated in only 12% yield. Several unidentified side products formed during the synthesis made isolation of the desired product difficult. Some of the side products may have resulted from the prolonged exposure of the reaction products to pyridine HCl formed during the reaction. However, formation of these side products was avoided by using methylphosphonoditriazolide (II, Figure 1) as the phosphorylating reagent. This reagent II is similar to the reagent used by Narang and coworkers[8] in the synthesis of deoxyoligonucleotide triesters.

Methods B and C use reagent (II) in the phosphorylation of the 3'-hydroxyl group of a 5'-protected nucleoside. The reagent was prepared *in situ* by stirring equimolar quantities of methylphosphonodichloridate, triethylamine, and triazole in anhydrous tetrahydrofuran at room temperature. The reaction of triazole with methylphosphonodicholoridate was slow and required 4 hr for completion. Formation of phenylphosphonoditriazolide was even slower and required 8 hr with stirring for completion. Both methyl and

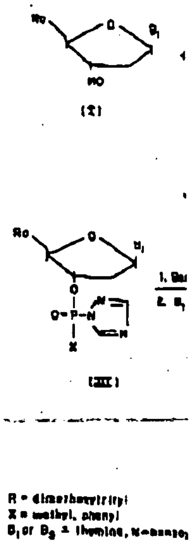

FIGURE 1. Synthesis of dinucl
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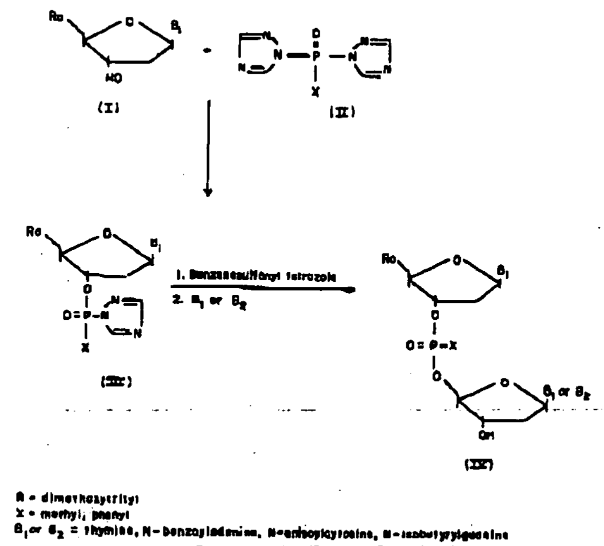

FIGURE 1. Synthesis of dinucleoside methyl and phenylphosphonate by one step procedure.

phenylphosphonoditriazolide were used directly, without isolation for the phosphorylation of the 3'-hydroxyl group of a 5'-dimethoxytritylated nucleoside. Phosphorylation of the 3'-hydroxyl group was rapid and reached completion within 30 minutes. Recently, we have shown[8] that in the synthesis of triesters that an intermediate similar to intermediate (III) did not phosphorylate the second nucleoside, and that further activation of this intermediate was necessary for an efficient second step phosphorylation. Further activation of intermediate (III), as described in Method B, was accomplished with triethylammonium benzenesulfonate. The activated intermediate thus obtained reacted slowly with the second nucleoside requiring 12 hr for 60% reaction. The yields of the isolated phosphonate diesters were in the range of 40-50%.

Although satisfactory yields of the dinucleoside phosphonates were obtained by Method B, synthesis by this method on a microscale (10-20 μmol) was unsatisfactory, and in each case the yield of the isolated product was very low (15-20%). In most cases, the second step of phosphorylation reached only 20-30% of completion. This may be due to partial hydrolysis of the activated intermediate (III). In order to improve the efficiency of the

second step of phosphorylation in microscale reactions, we investigated the activation of intermediate (III) by benzenesulfonyl tetrazole[9]. This reagent was prepared in situ and was added to intermediate (III) in the presence of the second nucleoside. A highly reactive phosphorylating intermediate was formed which reacted with the second nucleoside completely within one hr. By following this modification (Method C), oligonucleoside methylphosphonates were isolated in 60-70% yield (Table 1). This procedure was equally efficient for the synthesis of oligonucleoside phosphonates on a large scale (1-5 mmol). From the experiments described above it is clear that Method C is the method of choice for the synthesis of oligonucleotides carrying methyl or phenylphosphonate linkages.

Reaction products were readily separated by silica gel column chromatography using $CHCl_3$ containing 1% pyridine as the solvent. The columns were developed at a moderate pressure of 50 psi. The dinucleoside phosphonates eluted in 2-4% methanol while the tetranucleoside phosphonates eluted in 6-8% methanol. The reaction products from microscale synthesis were separated by silica gel preparative thin layer chromatography in solvent D and the desired product was isolated by elution with $CHCl_3$-MeOH-pyridine (7.8:2.0:0.2; v/v).

Both stepwise and blockwise elongation procedures were employed successfully for the synthesis of oligonucleoside methyl or phenylphosphonates. For

TABLE 1. Reaction conditions and isolated yields of the various dinucleoside methyl and phenylphosphonates.

[a]DMTr Comp. (equiv)	5'-OH Comp. (equiv)	[b]MePOCl$_2$ (equiv)	[c]PhPOCl$_2$ (equiv)	Triazole (equiv)	Et_3N (equiv)	[d]BST (equiv)	Nucleoside Phosphonate	[e]Yield
1. DMTr T (1.0)	dT (1.0)	1.0	-	2.5	2.5	1.0	DMTr T(pCH_3)T	65%
2. DMTr bzA (1.0)	dbzA (1.05)	1.0	-	2.6	2.5	1.1	DMTr bzA(pCH_3)bzA	62%
3. DMTr anC (1.0)	danC (1.05)	1.0	-	2.5	2.6	1.2	DMTr anC(pCH_3)anC	69%
4. DMTr ibG (1.0)	dibG (1.1)	1.05	-	2.8	2.8	1.3	DMTr ibG(pCH_3)ibG	58%
5. DMTr T (1.0)	dT (1.0)	-	1.0	2.5	2.5	1.1	DMTr T(pC_6H_5)T	60%
6. DMTr T(pCH_3)T (1.0)	dT(pCH_3)T (1.1)	1.15	-	3.0	3.0	1.8	Tetranucleoside phosphonate	53%
7. DMTr T(ClC_6H_4)T (1.0)	dT (1.0)	1.1	-	3.0	3.0	1.5	Trinucleoside phosphonate	50%
8. DMTr T(ClC_6H_4)T (1.0)	dT(ClC_6H_4)T (1.2)	1.15	-	3.0	3.0	1.1	Tetranucleoside phosphonate	62%

[a]Abbreviation for dimethoxytrityl group; [b]abbreviation for methylphosphonodichloridate; [c]abbreviation for phenylphosphonodichloridate; [d]abbreviation for benzenesulfonyl tetrazole; [e]isolated yields.

blockwise elongation. oligonucleoside phosphonate blocks of the appropriate size were synthesized in a stepwise manner. The dimethoxytrityl group was removed and the 5'-unprotected oligonucleotide was then condensed with 5'-protected oligonucleotide using Method C. For example, in the synthesis of the tetranucleoside methylphosphonate $d(Me_2O)TrT(pCH_3)T(pCH_3)T(pCH_3)T$, two dinucleotides, $d(Me_2O)TrT(pCH_3)T$ and $T(pCH_3)T$, were synthesized in a stepwise manner and coupled to give a tetranucleotide.

For the synthesis of oligonucleotides possessing the usual diester as well as phosphonate diester linkages, we have used a triester method[8] in combination with Method C. Synthesis of the tetranucleotide $d(Me_2O)TrT(ClC_6H_4)T(pCH_3)T(ClC_6H_4)T$ was accomplished by coupling two dinucleotides $d(Me_2O)TrT(ClC_6H_4)T$ and $T(ClC_6H_4)T$ in the presence of reagent (III) and benzenesulfonyl tetrazole as described in Method C. Removal of the protecting groups under appropriate conditions (described in Methods and discussed below) provided a tetranucleotide containing a single methylphosphonate linkage.

Oligonucleoside methylphosphonates were unstable in 1 M NaOH at room temperature. Under these conditions as much as 40% of the methylphosphonate diester linkage was hydrolyzed in one hr. Analysis of the cleaved products by silica gel tlc in the presence of the appropriate markers clearly showed that the hydrolysis of the methylphosphonate diester was random. Dithymidine phenylphosphonate, on the other hand, was more stable to hydrolysis under these conditions. Only a trace amount of hydrolysis (2-4%) was observed in one hour. However both phosphonate analogs of these oligonucleotides were resistant to hydrolysis by concentrated ammonium hydroxide at 36° for up to 16 hr; thereafter slow hydrolysis was observed. Treatment with concentrated ammonium hydroxide at elevated temperatures (>50°) resulted in rapid hydrolysis of both methyl and phenylphosphonate analogs. Since treatment with concentrated ammonium hydroxide at 36° for 16 hr does not produce any side reactions, these conditions were employed for the removal of p-chlorophenyl and amino protecting groups.

Both methyl and phenylphosphonate analogs were stable to mild acid. Treatment of these analogs with benzenesulfonic acid under the conditions used for removal of the dimethyoxytrityl group[9] produced no side products as judged by tlc. Removal of the dimethyoxytrityl group on a microscale (0.1 to 0.5 μmol) was achieved by treatment with pyridine-acetic acid-H_2O (3:14:1, v/v, pH 3.5) for 4 hr at room temperature. Even under these conditions no undesired products were formed.

To show that under the present set of conditions the major product of

synthesis carries a 5'→3' internucleotide linkage, we synthesized the dinucleotide d(Me$_2$O)TrT(pCH$_3$)T-OAC. The 3'-acetyl group was hydrolyzed by mild NaOH treatment at 4°. The dimethoxytrityl group was removed from the dinucleotide d(Me$_2$O)TrT(pCH$_3$)T by benzenesulfonic acid treatment as described in Methods. The dinucleotide was identical to the dinucleotide synthesized by using thymidine as shown by tlc and its interactions with snake venom phosphodiesterase and T4 polynucleotide kinase. Further evidence in support of this will come from studies of the separated isomers.

In dideoxynucleoside methyl or phenylphosphonates, the phosphorous atom is asymmetric so that two stereoisomers exist. This was shown by thin-layer chromatography and enzymatic studies. Thin-layer chromatography of the dinucleosidephosphonates gave two UV-absorbing spots moving close to each other indicating the presence of two stereoisomers. Separation, isolation, and identification of these two stereoisomers is presently under investigation.

No hydrolysis of 5'→3'-dithymidine methylphosphonate by spleen phosphodiesterase was detected after incubation for 24 hr at 37° using 50 times the amount of enzyme necessary to hydrolyze T-T in one hr. However, slow hydrolysis by snake venom phosphodiesterase was detected in the presence of excess enzyme. Only 50% of the methylphosphonate was hydrolyzed in 24 hr using 42 times the amount of enzyme needed to hydrolyze the same amount of T-T in 10 min. After 24 hr, no further hydrolysis was observed even after the addition of the same amount of enzyme initially used. Under identical conditions of enzyme excess, the phenylphosphonate analog of T-T was also hydrolyzed to 50% but at a much slower rate. These observations suggest that the snake venom phosphodiesterase may bind to and cleave only one stereoisomer. It is possible, however, that both stereoisomers bind to the enzyme equally well and that only one isomer interacts properly with the functional groups of the active site and can be cleaved. These alternatives are currently under study. Assuming that both T-T and T(pCH$_3$)T are hydrolyzed by the same active site, the slow hydrolysis of the preferred nonionic isomer may be due to the slow dissociation of the enzyme-product complex compared to T-T. Presumably the rapid hydrolysis of T-T is due to the fast dissociation of the enzyme-product complex. This phenomenon may be due to the electrostatic repulsion between the newly formed phosphomonoester and the negatively charged functional groups of the active site of the enzyme. In the case of nonionic analogs, this repulsive force is considerably lowered and thus reduces the rate of hydrolysis. If this is the case,

then the rate of hydrolysi
by the presence of these n
T-T was greatly reduced in
not shown). In an analogo
labeled oligo(dT)$_{15}$ (50 pm
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substrates in a snake veno
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Mixed ionic-nonionic
spleen phosphodiesterases.
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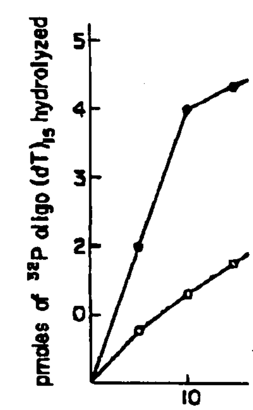

FIGURE 2. Rate of hydroly
phosphodiesterase in the p
oligo(dT)$_{15}$ (100 nM) in th
the hydrolysis of ^{32}P-olig
(100 μM) is shown by (o—

then the rate of hydrolysis of T-T and the oligonucleotides should be affected by the presence of these nonionic analogs. In fact the rate of hydrolysis of T-T was greatly reduced in the presence of equal amounts of T(pCH_3)T (data not shown). In an analogous experiment, the rate of hydrolysis of 5'-(^{32}P)-labeled oligo(dT)$_{15}$ (50 pmole) was markedly reduced in the presence of 5 nmol of T(pCH_3)T, while the addition of 5 nmol of T-T had very little effect upon the hydrolysis of the 5'-labeled oligo(dT)$_{15}$ as illustrated in Figure 2. These observations suggest that nonionic analogs may compete with the ionic substrates in a snake venom diesterase-catalyzed reaction. These results are preliminary and require further studies to determine the nature and mechanism of inhibition by these nonionic analogs.

Mixed ionic-nonionic analogs were also substrates for snake venom and spleen phosphodiesterases. For example, the tetranucleotide T-T(pCH_3)T-T was rapidly hydrolyzed to completion by snake venom phosphodiesterase to trinucleotide T-T(pCH_3)T (the trinucleotide had the same mobility on tlc as the chemically synthesized marker) as judged by tlc. However, further hydrolysis of the trinucleotide to thymidine was very slow and required 24 hr for 50% completion. Similarly the tetranucleotide T-T(pCH_3)T-T was rapidly hydrolyzed to T(pCH_3)T-T by spleen phosphodiesterase as judged by tlc. These observations suggest that the presence of a methylphosphonate

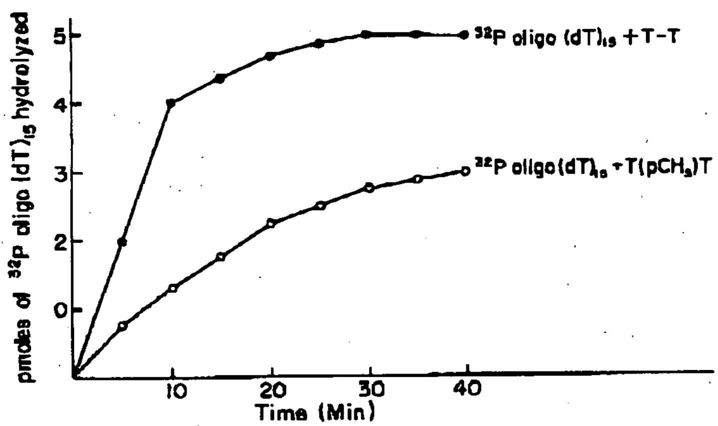

FIGURE 2. Rate of hydrolysis of 5'-(^{32}P)-labeled oligo(dT)$_{15}$ by snake venom phosphodiesterase in the presence of T-T or T(pCH_3)T. Hydrolysis of ^{32}P-oligo(dT)$_{15}$ (100 nM) in the presence of T-T (100 μM) is shown by (●—●) and the hydrolysis of ^{32}P-oligo(dT)$_{15}$ (100 nM) in the presence of T(pCH_3)T (100 μM) is shown by (o—o). Experimental details are described in Methods.

next to the phosphodiester does not affect the exonuclease activity.

In order to show that the nonionic analogs may also be substrates for other nucleic acid enzymes, we investigated the phosphorylation of T(pCH_3)T and T(pC_6H_5)T by T4-polynucleotide kinase in the presence of [γ-^{32}P]ATP. Our results showed that both analogs were phosphorylated and that the phosphorylation was several times slower than that of T-T. In each case the phosphorylated products were analyzed by PEI-cellulose thin layer chromatography which showed the presence of two radioactive spots as illustrated in Figure 3. Presumably the two radiolabeled spots correspond to the two stereoisomers. As may be seen in Figure 3, the slower migrating isomer of ^{32}pT(pCH_3)T contains more radioactivity than the faster moving isomer,

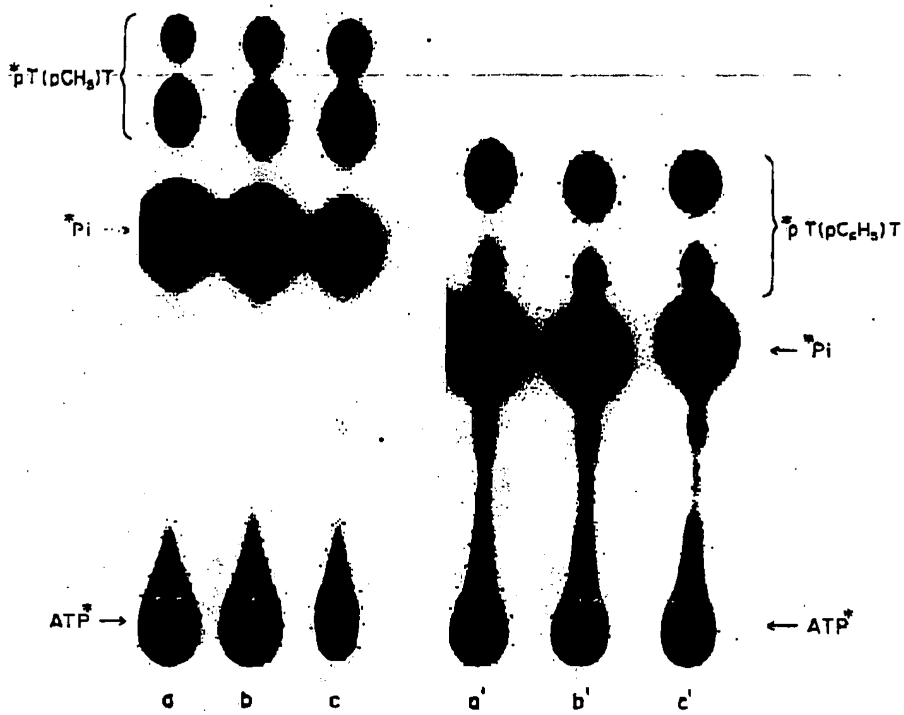

FIGURE 3. Autoradiogram of the phosphorylation of T(pCH_3)T and T(pC_6H_5)T by T4-polynucleotide kinase and [γ-^{32}P]ATP. Conditions of phosphorylations are described in Methods. Phosphorylation of T(pCH_3)T is shown on the left. a, b, and c are 2, 4, and 8 hr time intervals and a', b', and c' are 4, 8, and 12 hr time intervals.

suggesting that one isomer
polynucleotide kinase. In
contains more radioactivit
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Enzymatic studies on
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will be reported elsewhere.

CONCLUDING REMARKS

Availability of nonion
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EXPERIMENTAL

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All four nucleosides w
1-(H)-tetrazole, and dimeth
DEAE-cellulose plates were
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by water before use. Oligo

suggesting that one isomer is preferentially phosphorylated by the T4-polynucleotide kinase. In the case of $T(pC_6H_5)T$, the faster moving isomer contains more radioactivity than the slower migrating isomer. Phosphorylation of the methylphosphonate analog was several times faster than that of the phenylphosphonate analog. The difference in the rate of phosphorylation of the two analogs may be due to the bulky phenyl group in the phenylphosphonate analog.

Enzymatic studies on the nonionic analogs described above clearly show that they are ideal substrates for studying the mechanism of nucleic acid enzymes. Separation and characterization of the stereoisomers of these nonionic analogs will aid in precise understanding of their interaction with the various enzymes. Further studies along these lines are in progress and will be reported elsewhere.

CONCLUDING REMARKS

Availability of nonionic oligonucleotides as analogs of nucleic acids provides an opportunity to study the mechanism of action of nucleic acid enzymes. For the first time it is possible to synthesize pseudosubstrates for endonucleases which can be used to study DNA-protein complexes by physical and biochemical methods.

EXPERIMENTAL

Pyridine, tetrahydrofuran, and triethylamine were distilled and stored as previously described[8]. Thionyl chloride was refluxed over linseed oil for 10 hr and then distilled at atmospheric pressure in the presence of dry nitrogen stream. Methyl iodide was purified by filtration through a small plug of silica gel. Phenylphosphonodichloridate was purchased from Aldrich and was vacuum distilled before use. All condensation reactions were carried out by repeated evaporation *in vacuo* of a solution of component or components with added dry pyridine (at least four times) and the reaction flask was opened into a dry box which had a positive pressure of dry air[10].

All four nucleosides were obtained from Calbiochem. 1,2,4-Triazole, 1-(H)-tetrazole, and dimethyoxytrityl chloride were obtained from Aldrich. DEAE-cellulose plates were from Anal Tech. PEI-cellulose plates were from Brinkmann and were washed with 1.2 M pyridinium formate (pH 3.5) followed by water before use. Oligo$(dT)_{15}$ was purchased from P.L. Biochemicals.

Snake venom and spleen phosphodiesterases were purchased from Worthington Biochemicals. T4-polynucleotide kinase was isolated by a modified procedure of Panet *et al.*[11] and was completely free of contaminating exonucleases. [γ-^{32}P]ATP of high specific activity (1400 ci/mmol) was prepared by the procedure of Maxam and Gilbert[12] and was used without further purification. The dimethoxytritylated-amino protected nucleosides were prepared according to the published procedure[13]. Merck silica gel 60 F_{254} were used for thin layer chromatography in the following solvent systems: A, $CHCl_3$-EtOH (9.5:0.5: v/v); B, $CHCl_3$-EtOH (9:1; v/v); C, $CHCl_3$-EtOH (8.2; v/v); D, CH_3CN-H_2O (9:1; v/v); E, CH_3CN-H_2O (8.5:1.5; v/v); F, isopropanol-aqueous NH_4(d0.88)-H_2O (7:1:2; v/v).

Preparation of dimethyl methylphosphonate. This compound was prepared by the procedure described by Kosolopoff[14] except that the product was twice vacuum distilled (b.p. 80° at 25 mm) before use.

Preparation of methylphosphonodichloridate. To a stirred solution of thionyl chloride (150 ml) and pyridine (1.5 ml), dimethyl methylphosphonate (25 ml) was added slowly over a period of 2 hr. After the addition was complete, the solution was slowly brought to reflux and the refluxing was continued for a total of 24 hr. Excess thionyl chloride was removed by distillation at atmospheric pressure and the product was distilled twice under reduced pressure (b.p. 65° at 25 mm). Colorless low melting (m.p. 36°) white solid was isolated in 80% yield. Anal. Calcd. for CH_3POCl_2: Cl, 53.38. Found: Cl, 53.13.

Synthesis of the dinucleotide, d(Me_2O)TrT(pCH_3)T.

Method A

An anhydrous pyridine solution (10 ml) of 5'-O-dimethoxytritylthymidine (545 mg, 1.0 mmol) was added to a stirred pyridine solution (10 ml) of methylphosphonodichloridate (134 mg, 1.0 mmol). After 4 hr stirring at room temperature, the mixture was analyzed by silica gel tlc in solvents A (Rf 0.0) and B (Rf 0.18) which showed quantitative reaction. The second step of phosphorylation involved addition of an anhydrous pyridine solution (10 ml) of thymidine (243 mg, 1.0 mmol). The total reaction mixture was concentrated to 5.0 ml and stirred for 12 hr in the absence of moisture. Analysis of the reaction mixture by silica gel tlc in solvents B (Rf 0.66) and D (Rf 0.80) showed about 40% reaction. The reaction was terminated by cooling in dry ice-ethanol and adding $NaHCO_3$ (1 M, 20 ml) followed by $CHCl_3$ (100 ml). The oligonucleoside methylphosphonate was extracted in $CHCl_3$ (2 x 50 ml), concentrated under vacuum, dissolved in $CHCl_3$ (5 ml) contain-

ining 1% pyridine, and then c
pre-equilibrated with $CHCl_3$-1
pressure of 50 psi using a gr
and was monitored by tlc. Th
were pooled and concentrated
methylphosphonate (261 mg, 30
tion with hexane.

Method B

To a solution of 1,2,4-tr
(0.21 ml, 1.5 mmol) in anhydr
phosphonodichloridate (80 mg,
for 6 hr at room temperature a
a previously dried pyridine so
mg, 0.5 mmol) and the reaction
After 30 min at room temperatu
silica gel tlc in solvent B (R
lation. The second step of ph
the above reaction mixture an
sulfonic acid (264 mg, 1.65 mm
followed by an anhydrous pyrid
After 12 hr at room temperatur
gel tlc in solvents B (Rf 0.64
terminated and worked up as de
phonate diester (430 mg, 50% y
157-159° C. λ_{max} (EtOH), 267
C, 59.50; H, 5.54; N, 5.61. F

Method C

The first step of phospho
was on the same scale as descr
temperature, the phosphorylati
The second step of phosphoryla
sulfonyl tetrazole (0.6 mmol)
thymidine (145 mg, 0.6 mmol).
to 5 ml and kept at room tempe
tion mixture in solvents B (Rf
reaction. The reaction was te
A. The dithymidine methylphos
65% yield. m.p. 157-159° C.

ining 1% pyridine, and then chromatographed on silica gel column (2 x 30 cm) pre-equilibrated with $CHCl_3$-1% pyridine. The column was developed under a pressure of 50 psi using a gradient of methanol (0-5%) in the same solvent and was monitored by tlc. The fractions containing the required product were pooled and concentrated in the presence of pyridine. The dinucleoside methylphosphonate (261 mg, 30%) was isolated from the solvent by precipitation with hexane.

<u>Method B</u>

To a solution of 1,2,4-triazole (105 mg, 1.5 mmol) and triethylamine (0.21 ml, 1.5 mmol) in anhydrous tetrahydrofuran (10 ml) was added methylphosphonodichloridate (80 mg, 0.6 mmol). The reaction mixture was stirred for 6 hr at room temperature and then filtered. To the filtrate was added a previously dried pyridine solution of 5'-O-dimethoxytritylthymidine (272 mg, 0.5 mmol) and the reaction solution was concentrated <u>in vacuo</u> to 5 ml. After 30 min at room temperature the reaction mixture was analyzed by silica gel tlc in solvent B (Rf 0.18) which showed quantitative phosphorylation. The second step of phosphorylation was carried out by adding to the above reaction mixture an anhydrous pyridine (10 ml) solution of benzenesulfonic acid (264 mg, 1.65 mmol) and triethylamine (0.23 ml, 1.65 mmol) followed by an anhydrous pyridine solution of thymidine (145 mg, 0.6 mmol). After 12 hr at room temperature, the reaction mixture was analyzed by silica gel tlc in solvents B (Rf 0.64) and D (Rf 0.83). The reaction mixture was terminated and worked up as described above. The dinucleoside methylphosphonate diester (430 mg, 50% yield) was isolated in homogeneous form, m.p. 157-159° C. λ_{max} (EtOH), 267 (ε 19,987). Anal. Calcd. for $C_{42}H_{47}N_4O_{13}P$: C, 59.50; H, 5.54; N, 5.61. Found: C, 59.38; H, 5.46; N, 6.71.

<u>Method C</u>

The first step of phosphorylation of 5'-O-dimethyoxytritylthymidine was on the same scale as described in Method B. After 30 min at room temperature, the phosphorylation was complete as judged by silica gel tlc. The second step of phosphorylation was carried out by addition of benzenesulfonyl tetrazole (0.6 mmol) followed by a pyridine solution (10 ml) of thymidine (145 mg, 0.6 mmol). The total reaction mixture was concentrated to 5 ml and kept at room temperature for 1 hr. Silica gel tlc of the reaction mixture in solvents B (Rf 0.66) and D (Rf 0.79) showed about 80% reaction. The reaction was terminated and worked up as described in Method A. The dithymidine methylphosphonate was isolated in homogeneous form in 65% yield. m.p. 157-159° C. Anal. Calcd. for $C_{42}H_{47}N_4O_{13}P$: C, 59.50;

H, 5.54; N, 6.61. Found: C. 59.41; H, 5.43; N. 6.71.

Synthesis of dinucleotide, $d(Me_2O)TrT(pC_6H_5)T$. To a solution of 1,2,4-triazole (210 mg, 3.0 mmol) and triethylamine (0.43 ml, 3 mmol) in anhydrous tetrahydrofuran (25 ml) was added phenylphosphonodichloridate (0.14 ml, 1 mmol). The reaction mixture was stirred for 8 hr at room temperature and then filtered. To the filtrate a solution of 5'-O-dimethoxytritylthymidine (544 mg, 1 mmol) in anhydrous pyridine (10 ml) was added and the reaction mixture was concentrated to 10 ml. After 2 hr at room temperature, the reaction mixture was analyzed by silica gel tlc in solvent A (Rf 0.01) which showed over 90% phosphorylation. The second step of phosphorylation involved the addition of benzenesulfonyl tetrazole (1.5 mmol) followed by a pyridine solution (10 ml) of thymidine (284 mg, 1 mmol). The reaction mixture was concentrated to 10 ml and kept at room temperature for 6 hr. Silica gel tlc of the reaction mixture in solvents B (Rf 0.63) and C (Rf 0.86) showed about 70% reaction. Termination and work up of the reaction mixture was as described in Method A. The reaction products were separated on a silica gel column (2 x 30 cm) using a gradient of methanol (0-5%). Fractions containing the required product were pooled and concentrated in the presence of pyridine. The dithymidine phenylphosphonate, $d(Me_2O)TrT(pC_6H_5)T$, was isolated (545 mg, 60% yield) from the solvent by precipitation with hexane. λ_{max} (EtOH), 269 (ε 21,100): m.p. 171-174° C. Anal. Calcd. for $C_{47}H_{49}N_4O_{13}P$: C, 62.04; H, 5.39; N, 6.16. Found: C, 61.97; H, 5.37; N, 6.03.

Synthesis of $d(Me_2O)TrT(ClC_6H_4)T(pCH_3)T$. To an anhydrous solution of 1,2,4-triazole (8.4 mg, 124 µmole) and triethylamine (17 µl, 120 µmol) in tetrahydrofuran (200 µl) was added methylphosphonodichloridate (6.4 mg, 48 µmol). After six hr stirring at room temperature the reaction mixture was filtered and the filtrate was treated with an anhydrous solution of $d(Me_2O)TrT(ClC_6H_4)T$ (39.1 mg, 40 µmol) in pyridine (500 µl). The light yellow solution was concentrated to 100 µl and kept at room temperature for 30 minutes. Silica gel tlc of the reaction mixture showed complete phosphorylation. The second step involved an addition of benzenesulfonyl tetrazole (60 µmol) followed by a pyridine solution (200 µl) of thymidine (11.6 mg, 48 µmol). The reaction mixture was concentrated to 100 µl and kept at room temperature for 1 hr. Analysis of the reaction mixture by silica gel tlc showed that the trinucleotide, $d(Me_2O)TrT(ClC_6H_4)T(pCH_3)T$ was formed. The reaction mixture was separated by preparative tlc developed in solvent D. The trinucleotide was eluted from the thin layer plate with solvent D containing 2% pyridine and was isolated in 50% yield (24 mg).

Synthesis of $d(Me_2O)TrT(ClC_6H_4)T(pCH_3)T(ClC_6H_4)T$. A mixture of 1,2,4-triazole (8.4 mg, 120 μmol) and triethylamine (17 μl, 120 μmol) in anhydrous tetrahydrofuran (400 μl) was treated with methylphosphonodichloridate (6.2 mg, 46 μmol). After 6 hr at room temperature, the reaction mixture was filtered and the filtrate was treated with an anhydrous solution of $d(Me_2O)TrT(ClC_6H_4)T$ (33 mg, 40 μmol) in pyridine (200 μl). The combined solution was concentrated to 150 μl and was kept at room temperature. After 30 minutes it was analyzed by silica gel tlc. The second step of phosphorylation involved the addition of benzenesulfonyl tetrazole (45 μmol) followed by a pyridine solution (200 μl) of $T(ClC_6H_4)T$ (32.4 mg, 48 μmol). The total reaction mixture was concentrated to 100 μl and kept at room temperature for 1 hr. Silica gel tlc of the reaction mixture in solvents B (Rf 0.22) and C (Rf 0.46) showed 85% reaction. Termination and work up of the reaction mixture was as described in Method A. The reaction products were separated by preparative tlc developed in solvent D. The tetranucleotide was eluted from the thin layer plate with solvent D containing 2% pyridine and was isolated in 62% yield (41 mg).

Synthesis of $T(pCH_3)T$. $d(Me_2O)TrT(pCH_3)T$ was dissolved in ice-cold $CHCl_3$-*n*-butanol (9:1, v/v; 2 ml) and was treated with an equal volume of a 2% solution of benzenesulfonic acid in the same solvent. After 2 minutes at 4° C, the reaction was terminated by adding ice-cold $NaHCO_3$ (0.6 ml, 1 M) and the organic phase was recovered and evaporated under *vacuo*. The product (31 mg) was isolated from pyridine solution by precipitation into petroleum ether. λ_{max} (EtOH) 267 (ε 19,931). Anal. Calcd. for $C_{21}H_{29}N_4O_{11}P$: C, 46.23; H, 5.32; N, 10.27. Found: C, 46.20; H, 5.36; N, 10.33.

Synthesis of T-$T(pCH_3)T$. The trinucleotide, $d(Me_2O)TrT(ClC_6H_4)T(pCH_3)T$ (10 mg), was treated with concentrated ammonia (d 0.88) in pyridine (2 ml, 2:1; v/v) at 36° for 12 hr. Silica gel tlc in solvents D (Rf 0.31) and E (Rf 0.46) showed only one trityl positive spot. Ammonia and pyridine were removed by concentration *in vacuo* and the residue was treated with pyridine-acetic acid-H_2O (2 ml, 3:14:1; v/v, pH 3.5) for 90 minutes at room temperature. The solution was concentrated to dryness in vacuo and the residue was dissolved in H_2O (1 ml) and extracted with ether (2 x 3 ml). The aqueous layer was evaporated to dryness and dissolved in 1.0 ml of 5 mM Tris HCl (pH 7.5) and stored frozen.

Synthesis of T-$T(pCH_3)T$-T. The tetranucleotide, $d(Me_2O)TrT(ClC_6H_4)T$-$(pCH_3)T(ClC_6H_4)T$, was treated with concentrated ammonia followed by pyridinium acetate buffer pH 3.5 as described above for the trinucleotide.

Synthesis of $T(pC_6H_5)T$. For removal of dimethoxytrityl group from $d(Me_2O)TrT(pC_6H_5)T$, the procedure described for the synthesis of $T(pCH_3)T$ was used. After usual work up, the isolated product was homogeneous as judged by tlc in solvents D (Rf 0.82) and C (Rf 0.63). The product was isolated directly from the solvent by precipitation into hexane. λ_{max}(EtOH) 269 (ε 21,131). Anal. Calcd. for $C_{26}H_{31}N_4O_{11}P$: C, 51.40; H, 5.10; N, 9.22. Found: C, 51.32; H, 4.96; N, 9.63.

Enzyme Reactions

Snake venom phosphodiesterase-catalyzed hydrolysis of $T(pCH_3)T$ and $T(pC_6H_5)T$. Dithymidine methylphosphonate (5.76 OD_{267}, 300 nmol) was dissolved in 1 ml buffer (10 mM Tris HCl pH 8.0, 5 mM sodium phosphate, and 2 mM $MgCl_2$). Snake venom phosphodiesterase (1.5 U; this amount of enzyme is sufficient to completely hydrolyze 12 μmol of T-T at 37° in 2 hr) was added and the reaction was incubated at 37°. Aliquots of 100 μl were withdrawn at an interval of 2 hr and spotted on Whatman paper, and the paper was developed in solvent F. UV-absorbing spots were eluted from the paper with 1% aqueous NH_3 in 50% aqueous ethanol and their UV absorbance was measured.

For the hydrolysis of dithymidine phenylphosphonate, the procedure and the quantities of various components were the same as described above.

Hydrolysis of 5'-[^{32}P]labeled oligo$(dT)_{15}$ + T-T by snake venom phosphodiesterase in the presence and absence of $T(pCH_3)T$. 5'-[^{32}P]-labeled oligo $(dT)_{15}$ (50 pmol, 1.1 x 10 cpm/pmol) and $T(pCH_3)T$ (5 nmol, 0.01 OD_{267}) was dissolved in 40 μl of buffer (10 mM Tris HCl pH 8.0, 5 mM sodium phosphate, and 2 mM $MgCl_2$) and treated with snake venom phosphodiesterase (10 μl of 1.0 mg/ml stock solution). The reaction mixture was incubated at room temperature and 5 μl aliquots were withdrawn at 5 minute intervals and applied to a DEAE-cellulose thin layer plate. The plate was developed in homomix-IV of Jay et al.[15] The radioactive spots were located by autoradiography. The radioactive band corresponding to oligo$(dT)_{15}$ was scraped and counted. Decrease in radioactivity with time provided a measure of oligo$(dT)_{15}$ hydrolyzed. Similarly in a parallel experiment $T(pCH_3)T$ was replaced by the same amount of T-T.

Phosphorylation of $T(pCH_3)T$ and $T(pC_6H_5)T$ by T4-polynucleotide kinase and [γ-^{32}P]ATP. The dinucleotides, $T(pCH_3)T$ or $T(pC_6H_5)T$ (200 pmol), and [γ-^{32}P]ATP (710 pmol, 2000 cpm/pmol of the ATP) were suspended in 10 μl of buffer (50 mM Tris HCl pH 9.0, 5 mM DTT, 2 μM spermidine, and 10 mM $MgCl_2$). The reaction mixture was heated at 95° for 2 minutes and then cooled to 37°.

The T4-polynucleotide kinase (1 μl, 1 unit/μl) was added and the reaction was incubated at 37°. Aliquots of 2 μl were withdrawn at intervals of 2, 4, and 12 hr in the case of T(pCH_3)T and 4, 8, and 24 hr in the case of T(pC_6H_5)T. The aliquots were directly applied to prewashed PEI-cellulose thin layer plates and the plates were developed in 1.5 M pyridinium formate pH 3.5. Figure 3 shows the autoradiogram of the phosphorylated products.

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This work is dedicated to the late Professor G.W. Kenner.

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Preparation of triple-block DNA pol

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ABSTRACT
The construction of
tives containing novel t
described. The protocol
thesis of a heterogenous
$dA_{\overline{40}}dC_{\overline{40}} \cdot dT_{\overline{40}}dC_{\overline{40}}$, using
minal transferase. The
earized and dG-tailed ve
form E. coli. Triple-bl
$dG_{g}dT_{t}dC_{c}$, characterized
the Bam HI site of pBR32
promoter regions in pRW2
inserted into the Sma I
by cleavage with Sma I si
recognition site. The a
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polymers in essentially
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stable hairpin structures
and appear to replicate

INTRODUCTION

Biochemical and biop
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VIROLOGY 42, 421–428 (1970)

The Antiviral Activity of Thiophosphate-Substituted Polyribonucleotides *in Vitro* and *in Vivo*[1]

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As shown before for the alternating poly r(A-U) (riboadenylic-ribouridylic acid), substitution of thiophosphate for phosphate in the alternating poly r(I-C) (riboinosinic-ribocytidylic acid) resulted in a significant increase of its ability to induce *in vitro* resistance to virus infection and interferon production *in vitro* and *in vivo*. The antiviral activity of the partially substituted polynucleotides poly r(A_sU) and poly r($_s$I-C) was intermediate between the antiviral activities of the unsubstituted polynucleotides poly r(A-U) and poly r(I-C) and those of the completely substituted analogs poly r($_sA_sU$) and poly r($_sI_sC$). The thiophosphate-substituted polyribonucleotides showed an increased resistance to degradation by nucleases present in fetal calf serum. The nucleolytic activity of the serum resembled pancreatic rather than T1 ribonuclease. The increased resistance to breakdown by ribonuclease and serum paralleled an increased capacity to confer cellular resistance to virus infection, suggesting that protection against premature enzymatic degradation might underly the increased antiviral activity of the thiophosphate analogs *in vitro* and *in vivo*.

INTRODUCTION

Various synthetic polyanions, including polycarboxylates, polyphosphates, polythiophosphates, and polysulfates, stimulate interferon production *in vitro* and *in vivo* (as reviewed by De Clercq and Merigan, 1970). Their antiviral activity largely depends on the presence of a stable long-chain backbone on which free negative charges occur in a tightly packed and regular sequence (Merigan and Finkelstein, 1968; De Somer *et al.*, 1968; De Clercq and Merigan, 1969; De Clercq *et al.*, 1970a). Double-stranded polyribonucleotides such as the polyriboinosinic acid/polyribocytidylic acid homopolymer pair (poly rI)·(poly rC), are the most potent interferon inducers on a weight basis (Field *et al.*, 1967, 1968; Hilleman, 1970). Substitution of sulfur for oxygen in the phosphate linkages of the double-stranded polyribonucleotide poly r(A-U) (an alternating copolymer of riboadenylic acid and ribouridylic acid) has recently been shown to cause a significant increase of its interferon-inducing capacity (De Clercq *et al.*, 1969). In this report we present findings on the antiviral activity of the partially thiophosphate-substituted poly r(A-U), the partially and completely thiophosphate-substituted analogs of poly r(I-C) (an alternating copolymer of riboinosinic acid and ribocytidylic acid) and relate their antiviral activity to their degradability by purified enzymes or serum.

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[2] Fellow of the Damon Runyon Memorial Fund for Cancer Research and "Aangesteld Navorser" of the Belgian N.F.W.O. (Nationaal Fonds voor Wetenschappelijk Onderzoek).

MATERIALS AND METHODS

Preparation of the polynucleotides and their thiophosphate-substituted analogs. The prep-

aration of poly r(A-U)[3,4] poly r(A;U) and poly r(;A;U) has been described (Eckstein and Gindl, 1970). A first preparation of poly r(I-C) [poly r(I-C)I] was synthesized without a template employing the DNA-dependent RNA-polymerase prepared according to Zillig *et al.* (1966); a second preparation [poly r(I-C)II] was prepared with poly d(I-C) as a template (Sternbach and Eckstein, 1970). Poly r(;I-C) was synthesized on poly d(I-C) in analogy to poly r(I-C)II. Poly r(;I;C) was prepared in analogy to poly r(I-C)I without a template.

The homopolymer pair (poly rI)·(poly rC) was prepared by mixing polyinosinic acid and polycytidylic acid (both purchased from Miles Laboratories, Elkhart, Indiana) in equimolar concentrations in unbuffered saline (0.15 M NaCl) and allowing them to stand at 25° for at least 2 hours. Duplex formation was evidenced by hypochromicity and finding of the appropriate transition mid point T_m:57° in 0.1 M Na^+ citrate buffer. The (poly rI)·(poly rC) preparation employed in the rabbit experiments was obtained with homopolynucleotides purchased from P-L Biochemicals, Inc., Milwaukee, Wisconsin. (Poly rI)·(poly rC) was kept frozen in aliquots at −20° at 1.0 mg/ml in saline. Poly r(A-U), poly r(I-C), and their thiophosphate analogs were stored at −20° at 20 µg/ml in saline.

[3] Abbreviations for the polynucleotides are those proposed by the IUPAC-IUB (1965) Rules in *J. Biol. Chem.* 241, 527 (1966), and revised by a recent communication of the National Research Council (Office of Biochemical Nomenclature) (January 20, 1969). The abbreviations for the thiophosphate-substituted polynucleotides are those suggested by Dr. W. E. Cohn (Director of the National Academy of Sciences–National Research Council, Office of Biochemical Nomenclature, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

[4] Physical-chemical properties: Sedimentation ($s_{20,w}$) values and thermal stability (T_m) values for poly r(A-U) (preparations I and II) and poly r(;A;U) (preparations I and II) as described before (De Clercq *et al.*, 1969); molecular weight (MW^{s-d}): poly r(A-U)II, 128,200; poly r(A;U), 105,200; poly r(;A;U)II, 30,000; poly r(I-C)I, 35,000; poly r(I-C)II, 87,000; poly r(;I;C), 294,000; T_m (°C): poly r(I-C)I, 47; poly r(I-C)II, 47; poly r(;I-C), 50; poly r(;I;C), 51 (all T_m values determined in 0.1 M Na^+ citrate buffer).

Cell culture. Human skin fibroblasts (HSF) and RK 13 cells (a continuous rabbit kidney cell line) were grown and maintained in 60-mm Falcon plastic petri dishes as described elsewhere (Merigan *et al.*, 1966). Cell cultures were incubated in minimal Eagle's medium (MEM) with added penicillin G (100 units/ml) and streptomycin (100 µg/ml). Ten percent calf serum was employed during the growth of the cells; serum was omitted during the antiviral activity experiments.

Cellular resistance to virus infection and interferon production in vitro. Interferon production and cellular resistance to bovine vesicular stomatitis virus (VSV) (Indiana strain) were measured in HSF as described previously (De Clercq and Merigan, 1969).

To follow the *persistence of cellular resistance* to VSV in HSF, cells were exposed to various concentrations of the polymers for 20 hours at 37°; the polymer was then removed, the cells were washed with MEM and further incubated with polymer free MEM until challenge with VSV.

Interferon was characterized by its sensitivity to trypsin (0.25 mg/ml, 1 hour, 37°), resistance to pancreatic ribonuclease [40 µg/ml, 1 hour, 37°, 10^{-3} M ethylenediaminetetraacetic acid (EDTA)] and lack of activity in heterologous (mouse L 929) cells.

Interferon production in vivo. Albino rabbits weighing 1.5–2.0 kg were injected intravenously with 4 µg of the polymer (in 1–4 ml MEM). Blood samples were taken 1.5, 3, 6, 12, and 24 hours later and the serum assayed for interferon with the plaque reduction technique in RK 13 cell cultures employing VSV as the challenge virus. Interferon was characterized as described above.

Sensitivity to fetal calf serum. Different polymer concentrations (0.4 or 0.04 µg/ml) in MEM were incubated for 1 hour at 37° and then exposed to fetal calf serum (obtained from Microbiological Associates Inc., Bethesda, Maryland) for 2 hours 37°. (Final concentration of fetal calf serum: 2, 10, or 50%). Residual antiviral activity of the mixtures was measured by VSV plaque reduction in HSF.

Sensitivity to pancreatic ribonuclease. The polymers were diluted in MEM + 10^{-3} M EDTA to 0.04 µg/ml and exposed to various concentrations of pancreatic RNase (bovine pancreatic ribonuclease A, 5 × crystallized,

urchased from Sigma Chemical Company, St. Louis, Missouri) for 1 hour at 37°. The residual antiviral activity was determined by incubating the samples with HSF and measuring VSV plaque reduction as described elsewhere (De Clercq *et al.*, 1969).

Sensitivity to T1 ribonuclease. Polymer dilutions in MEM (without EDTA) at 0.004 μg/ml were preincubated at 37° for 1 hour. T1 RNase (Worthington Biochemical Corporation, Freehold, New Jersey) was added in various concentrations and the mixtures further incubated for 2 hours at 37°. Residual antiviral activity of the samples was measured by VSV plaque reduction in HSF.

RESULTS

Induction of Cellular Resistance to Virus Infection in Vitro: Polymer Dilutions Prepared and Preincubated in MEM at Different Temperatures

Prior incubation in MEM at 37° before exposure to the cell cultures has recently been shown to increase the antiviral activity of both RNA and DNA alternating copolymers and homopolymer pairs (De Clercq *et al.*, 1970b). Upon preincubation in MEM at 37°, poly r(A-U) and poly r(I-C) reduced VSV plaque formation in HSF at a 10^5–10^6-fold lower concentration than when the polymers had been preincubated in MEM at 0° (Table 1). There was no significant activation when poly r(A-U) and poly r(I-C) were held at 25° before exposure to the cells (Table 1). Addition of 10^{-3} *M* EDTA following the 37° incubation step reversed the thermal activation of both copolymers completely.

The thiophosphate analogs poly r($_s$A$_s$U) and poly r($_s$I$_s$C) were considerably more active than the unsubstituted parent compounds when preincubated in MEM at 0° or 25° (Table 1). There was a slight increase in antiviral activity of the polythiophosphates preincubated at 25° over the precooled (0°) polymers. Preincubation at 37° however, did not further increase the virus plaque-reducing efficiency of poly r($_s$A$_s$U) and poly r($_s$I$_s$C), as compared to preincubation at 25°. The antiviral activity of the preheated (37°) polythiophosphates returned to the level of the precooled (0°) polymers when 10^{-3} *M* EDTA was added after the 37° incubation step. The partially substituted polymers poly r(A$_s$U) and poly r($_s$I-C) (not reported in Table 1) showed an intermediate behavior, as compared to poly r(A-U) and poly r($_s$A$_s$U) or poly r(I-C) and poly r($_s$I$_s$C), in that they reduced virus plaque formation at a 10–100-fold lower concentration than the unsubstituted polymers (when preincubated at 25°). Their antiviral activity shifted about 10^4 times after preincubation at 37° and could not be reversed completely upon addition of 10^{-3} *M* EDTA.

TABLE 1

EFFECT OF PREINCUBATION OF THE POLYNUCLEOTIDES AND THEIR THIOPHOSPHATE-SUBSTITUTED ANALOGS AT DIFFERENT TEMPERATURES ON THEIR ABILITY TO CONFER RESISTANCE TO VESICULAR STOMATITIS VIRUS IN HUMAN SKIN FIBROBLASTS[a]

Polymer	Minimal inhibitory concentration[b] (μg/ml) Polymer preincubated in MEM for 2 hours at			
	0°	25°	37°	37° (10^{-3} *M* EDTA)[c]
Poly r(A-U) II	>1	4	0.000004	≥0.4
Poly r($_s$A$_s$U) II	0.001	0.001	0.0001	0.001
Poly r(I-C) II	>10	>4	0.00004	>1
Poly r($_s$I$_s$C)	0.001	0.0001	0.0001	0.001
(Poly rI)·(poly rC)	0.004	0.004	0.00004	0.04

[a] Serial (1 to 10) dilutions of the polymer prepared and preincubated in MEM at the given temperature and immediately applied to the cells.

[b] Reducing VSV plaque formation by 50%.

[c] Added at the end of the preincubation period.

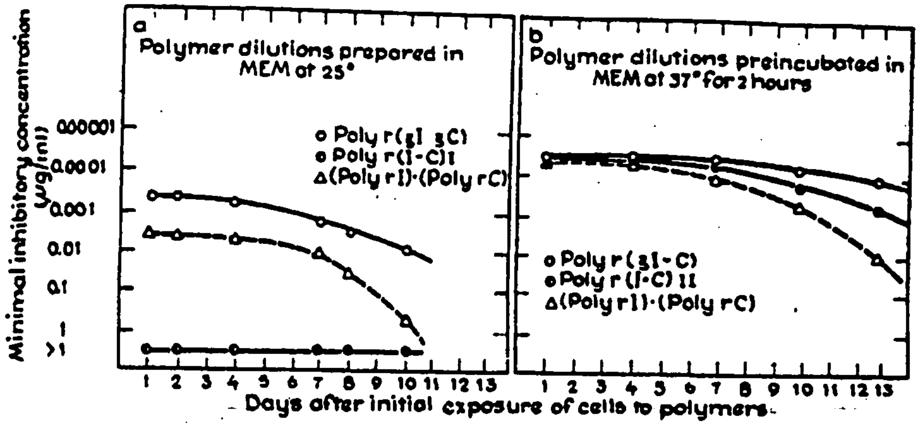

FIG. 1. Persistence of cellular resistance to vesicular stomatitis virus in human skin fibroblasts. Serial (1 to 10) dilutions of the polymers were prepared in MEM at 25° (panel a) or prepared and preincubated in MEM at 37° (panel b) and then applied to the cell monolayers. At various time intervals thereafter the cells were exposed to virus challenge as described in Materials and Methods. Minimal inhibitory concentration indicates the lowest polymer concentration which reduces virus plaque formation by 50%.

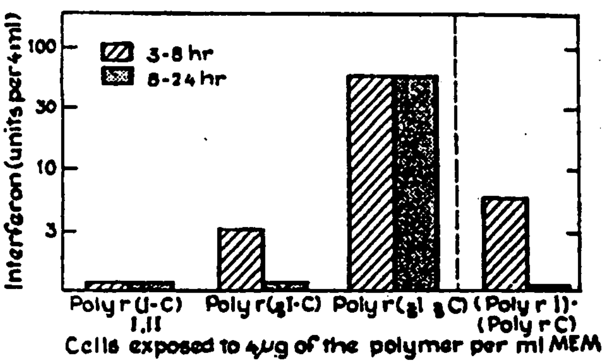

FIG. 2. Interferon production in human skin fibroblasts. Polymers exposed to the cells for 3 hours and interferon production measured in the subsequent 3–8 hour and 8–24 hour time periods.

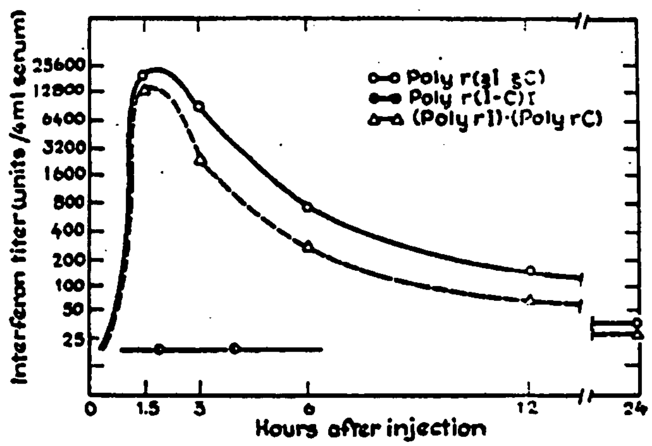

FIG. 3. Interferon production in the rabbit. Serum interferon titers measured at several times after intravenous injection of 4 µg of the polymer (in 1–4 ml MEM) per rabbit. The polymer dilutions were prepared at 25°.

Persistence of Cellular Resistance to Virus Infection in Vitro

When cell cultures were challenged with VSV at several times after the initial exposure to either poly r(I-C), poly r($_s$I-C), poly r($_s$I$_s$C), or (poly rI)·(poly rC), resistance to virus plaque formation was maintained up to day 7 after treatment and diminished thereafter (Fig. 1). Resistance to virus infection decreased more rapidly for (poly rI)·(poly rC) than for poly r($_s$I-C), poly r($_s$I$_s$C), and the preheated (37°) poly r(I-C). When preincubated at 25°, poly r(I-C) did not reduce virus plaque formation at the concentration tested (Fig. 1A).

Interferon Production in Vitro

The partially and completely thiophosphage-substituted analogs of poly r(I-C) stimulated interferon production in HSF under conditions the unmodified compound did not (Fig. 2). Significantly more interferon was obtained with poly r($_s$I$_s$C) than with (poly rI)·(poly rC). Poly r($_s$I$_s$C) continued to stimulate interferon production during the 8–24 hour incubation period, whereas (poly rI)·(poly rC) did not.

Interferon Production in Vivo

Poly r($_s$A$_s$U) has been reported to have a significantly (40-fold) greater interferon inducing capacity than poly r(A-U) in the rabbit (De Clercq *et al.*, 1969). A similar increase in interferon production in rabbits

was observed following substitution of thiophosphate for phosphate in poly r(I-C) (Fig. 3). At a concentration of 4 μg per rabbit, poly r($_s$I$_s$C) produced up to 20,000 units and poly r(I-C) less than 20 units of interferon per 4 ml of serum. Under similar conditions (1.5 hr after intravenous injection) 4 μg of poly r($_s$I-C) stimulated the production of 40 units/4 ml of circulating interferon. Preheating at 37° (in MEM) did not markedly increase the interferon inducing capacity of poly r(I-C), poly r($_s$I$_s$C), or (poly rI)·(poly rC) in the rabbit.

With both (poly rI)·(poly rC) and poly r($_s$I$_s$C) peak serum interferon titers were reached in the rabbit at 1.5 hour after intravenous injection (Fig. 3). Interferon titers decreased steadily from 1.5 hour to 24 hours. The kinetics of interferon production with poly r($_s$I$_s$C) and (poly rI)·(poly rC) were essentially the same, suggesting a similar production mechanism for regular and thiophosphate-substituted polynucleotides.

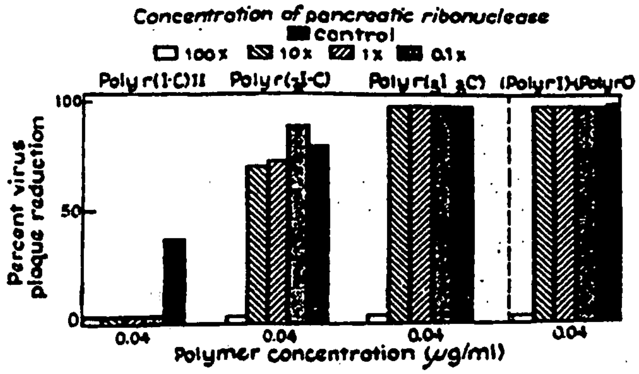

FIG. 5. Sensitivity to pancreatic ribonuclease as measured by ability to confer cellular resistance to vesicular stomatitis virus in human skin fibroblasts. Ratios of ribonuclease to polymer concentration on a weight basis were 100, 10, 1, and 0.1. Pancreatic ribonuclease alone did not affect the virus plaque number at the concentrations used.

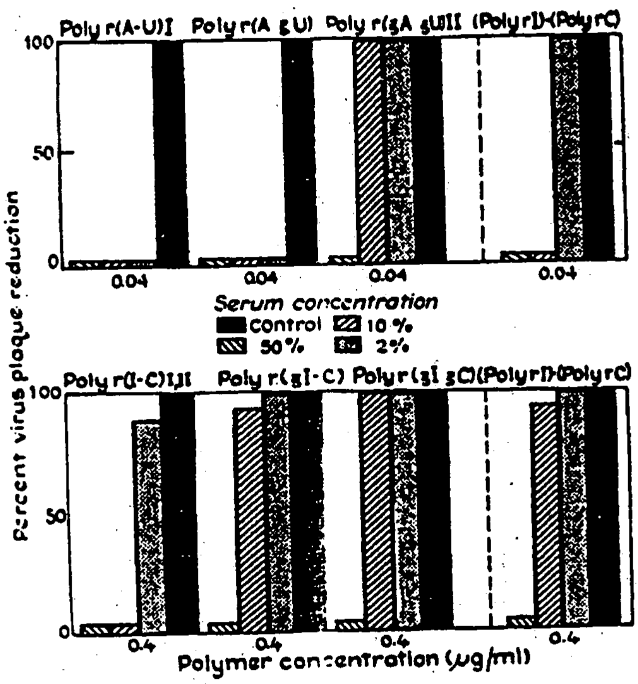

FIG. 4. Sensitivity to fetal calf serum as measured by ability to confer cellular resistance to vesicular stomatitis virus in human skin fibroblasts. Because fetal calf serum itself caused a reduction (up to 40%) or increase (up to 50%) of the virus plaque number at the serum concentrations used (50% and 10%, respectively) as compared to control cell cultures which had been incubated with MEM with 2% serum or without serum, plaque reduction is expressed in percentage of controls treated with the corresponding concentration of serum.

Sensitivity to Fetal Calf Serum

Human serum as well as chicken and fetal calf serum abolishes the pyrogenic activity of (poly rI)·(poly rC), and this inhibitory effect is thought to be enzymatic in origin (Nordlund *et al.*, 1970). Serum nucleases might also inactivate the interferon inducing capacity of (poly rI)·(poly rC) as both pyrogenicity and interferon-inducing capacity are closely related phenomena, depend on the complementary base-paired complex formation of the molecule (Lindsay *et al.*, 1969). Premature degradation by serum (or plasma) nucleases may be responsible for the short-lived interferon titers and antiviral activity of polynucleotides *in vivo*.

Poly r(A-U), poly r(I-C), and their thiophosphate analogs were exposed to fetal calf serum under conditions similar to those employed to test their sensitivity to enzymatic degradation. All polymers were heat-activated through preincubation at 37° in MEM for 1 hour. EDTA was omitted. Poly r(A$_s$U) proved as sensitive and poly r($_s$A$_s$U) considerably less sensitive to fetal calf serum than poly r(A-U) (Fig. 4). Poly r($_s$A$_s$U) was even less sensitive to serum inactivation than (poly rI)·(poly rC). Both poly r($_s$I-C) and poly r($_s$I$_s$C) were more resistant to fetal calf serum than poly r(I-C) but equally resistant as (poly rI)·(poly rC).

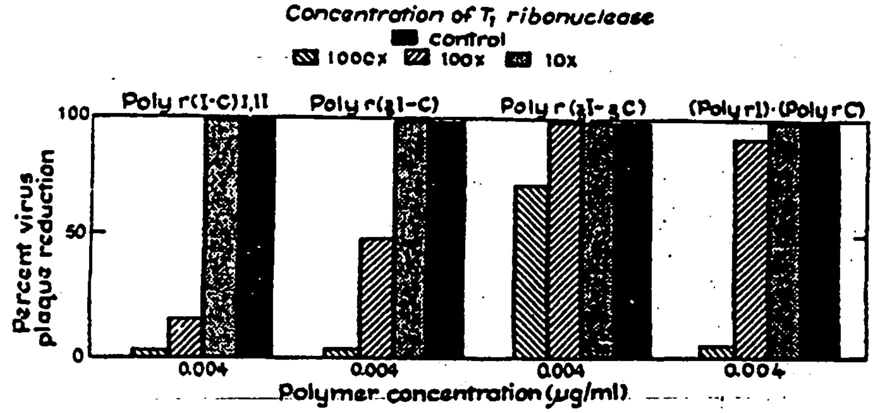

FIG. 6. Sensitivity to T1 ribonuclease as measured by ability to confer cellular resistance to vesicular stomatitis virus in human skin fibroblasts. Ratios of ribonuclease to polymer concentration on a weight basis were 10,000, 1000, 100, and 10. T1 ribonuclease alone did not affect the virus plaque number at the concentrations used.

Sensitivity to Pancreatic Ribonuclease

As demonstrated previously with poly r(ₛAₛU) (De Clercq *et al.*, 1969), poly r(ₛIₛC) proved markedly more resistant to degradation by pancreatic ribonuclease than its unmodified parent compound (Fig. 5). The partially substituted analogs poly r(AₛU) and poly r(ₛI-C) showed an interesting difference in sensitivity to pancreatic RNase as compared to their parent compounds: poly r(AₛU) did not differ significantly in sensitivity to pancreatic RNase from poly r(A-U) (not shown in Fig. 5), whereas poly r(ₛI-C) was significantly more resistant than poly r(I-C) and equally resistant as the completely substituted poly r(ₛIₛC) (Fig. 5). Both poly r(ₛI-C) and poly r(ₛIₛC) resembled (poly rI)·(poly rC) in sensitivity to pancreatic RNase.

Sensitivity to T1 Ribonuclease

Figure 6 shows that (1) poly r(I-C) was more sensitive to T1 ribonuclease than (poly rI)·(poly rC) (which is consistent with the findings reported by Krakow and Karstadt, 1967) and that (2) complete substitution of thiophosphate for phosphate [in poly r(ₛIₛC)] rendered poly r(I-C) significantly more resistant to T1 ribonuclease whereas partial substitution [in poly r(ₛI-C)] did not.

DISCUSSION

The completely thiophosphate-substituted analog of poly r(I-C) [poly r(ₛIₛC)] showed an antiviral activity like the homopolymer pair (poly rI)·(poly rC), used as a reference in our experiments, but surpassed the activity of the unsubstituted poly r(I-C) by several orders of magnitude. The low activity of poly r(I-C) was surprising as others found poly r(I-C) and (poly rI)·(poly rC) similar in activity (Colby and Chamberlin, 1969; Hilleman, 1970). The poly r(I-C) preparations employed in our experiments were obtained by an unprimed polymerization of ITP and CTP or by polymerization with poly d(I-C) as a template (Sternbach and Eckstein, 1970). Both preparations had an appropriate nearest neighbor analysis and a thermal stability of 47° (in 0.1 *M* Na^+) (compared to 60° for the preparation used by Colby and Chamberlin, 1969), below the threshold thermal stability suggested as critical for interferon production and full antiviral activity (De Clercq and Merigan, 1969).

Poly r(ₛAₛU) and poly r(ₛIₛC) were somewhat more active than (poly rI)·(poly rC) in inducing cellular resistance to virus infection in human skin fibroblasts when preincubated at 25° (Table 1); poly r(ₛIₛC) stimulated more interferon production than (poly rI)·(poly rC) in human cells (Fig. 2) and the cellular resistance offered by poly r(ₛI-C) and poly r(ₛIₛC) persisted for a longer time than with (poly rI)·(poly rC) (Fig. 1). The kinetics of interferon production by poly r(ₛIₛC) and (poly rI)·(poly rC) in the rabbit were almost identical. The similar patterns of antiviral activity of (poly rI)·(poly rC)

and the thiophosphate-substituted poly (I-C) and poly r(A-U) suggest that their mode of action is identical and that introduction of sulfur in the phosphate groups does not diminish the affinity of polynucleotide interferon inducers for the cellular receptor or triggering site for interferon production, postulated by Colby and Chamberlin (1969) and De Clercq and Merigan (1969).

Substitution of thiophosphate for phosphate rendered both poly r(I-C) and poly r(A-U) more resistant to degradation by endonucleases (pancreatic ribonuclease, T1 ribonuclease, as measured by antiviral activity (Figs. 5 and 6; De Clercq *et al.*, 1969) or acid-insoluble radioactivity (Eckstein and Gindl, 1970). The increased resistance of the thiophosphate-substituted polynucleotides to degradation by pancreatic and T1 RNase (Figs. 5 and 6) closely paralleled their degree of antiviral activity in human skin fibroblasts. For the poly r(I-C) species, resistance to enzymatic breakdown and antiviral activity increased in the order poly r(I-C), poly r(ₛI-C), and poly r(ₛIₛC).

The partially thiophosphate-substituted polynucleotides showed a paradoxically different behavior in their susceptibility to pancreatic RNase. Poly r(ₛI-C) was significantly more resistant to pancreatic RNase than poly r(I-C) whereas poly r(AₛU) did not differ from poly r(A-U) in sensitivity to pancreatic RNase. These differences might be explained by the base specificity of pancreatic RNase which cleaves RNA between the phosphate attached to the 3′-OH group of uracil or cytosine nucleosides and the 5′-OH group of whatever nucleotide is next. In poly r(ₛI-C) the phosphate at the 3′-OH end of cytidine is replaced by thiophosphate and is therefore expected to be more resistant. In poly r(AₛU) the phosphate at the 3′-OH end of uridine is intact whereas the phosphate group at the 5′-OH end of uridine has been substituted by thiophosphate: this arrangement would not be expected to show increased resistance to pancreatic RNase.

If the increased resistance of the thiophosphate analogs to enzymatic breakdown depends on the internucleotide bond for which the enzyme has its specific affinity, substitution of thiophosphate for phosphate at the 5′-OH end of inosine [in poly r(ₛI-C)] should not increase the resistance to T1 ribonuclease, whereas further substitution at the 3′-OH end of inosine [in poly r(ₛIₛC)] should do so. T1 ribonuclease is known to split nucleotide bonds adjacent to the 3′-OH end of guanosine (and guanosine derivatives inosine and xanthosine) (see, e.g., Whitfeld and Witzel, 1963; Egami *et al.*, 1964). Our results have shown that poly r(ₛI-C) was somewhat more resistant to T1 ribonuclease than poly r(I-C) but that further modification to poly r(ₛIₛC) resulted in a more significant increase in resistance (Fig. 6).

It is likely that the increased antiviral activity of polynucleotides seen in tissue culture following thiophosphate substitution is due to protection of the polynucleotides against premature enzymatic degradation. The increased interferon production *in vivo* might be related to an increased resistance to nucleases in the serum. Figures 4, 5, and 6 show that introduction of thiophosphate groups in poly r(A-U) and poly r(I-C) lead to a parallel increase in the resistance to degradation by fetal calf serum, pancreatic RNase and T1 RNase and that the partially substituted polymers poly r(AₛU) and poly r(ₛI-C) differed in sensitivity to fetal calf serum from their parent compounds as they did in sensitivity to pancreatic RNase. These findings suggest that the inactivating effect of serum is mediated by nuclease with the specificity of pancreatic RNase. The role of nucleases in the inhibitory effect of serum is further supported by the findings that polyribonucleotides [e.g., (poly rI)·(poly rC)] became more acid soluble the longer they were incubated with fetal calf serum (our unpublished data), human or rabbit serum (Nordlund *et al.*, 1970).

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Biochemical and Biological Effects of Nonionic Nucleic Acid Methylphosphonates†

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ABSTRACT: Oligodeoxyribonucleoside methylphosphonates with base sequences complementary to the anticodon loop of $tRNA^{Lys}$ and to the –ACCA–OH amino acid accepting stem of tRNA were prepared by chemical synthesis. Oligodeoxyadenosine methylphosphonates form stable, triple-stranded complexes with both poly(U) and poly(dT). These analogues selectively inhibit cell-free aminoacylation of $tRNA^{Lys}_{E.coli}$ but have no effect on aminoacylation of $tRNA^{Lys}_{rabbit}$. The extent of inhibition is temperature dependent and parallels the ability of the oligomer to bind to poly(U), which suggests that inhibition occurs as a result of oligomer binding to the –UUUU– anticodon loop of $tRNA^{Lys}_{E.coli}$. The failure of the oligodeoxyadenosine methylphosphonates to inhibit $tRNA^{Lys}_{rabbit}$ aminoacylation suggests that there may be a difference between the structure of $tRNA^{Lys}$ or its interaction with aminoacyl synthetase in the *Escherichia coli* and rabbit systems. The oligodeoxyadenosine analogues also effectively inhibit polyphenylalanine synthesis in cell-free translation systems derived from both *E. coli* and rabbit reticulocytes. The extent of inhibition parallels the T_m values of the oligo(A) phosphonate–poly(U) complexes and suggests that the inhibition [illegible] consequence of complex formation with the poly(U) [illegible]. Tritium-labeled oligodeoxyribonucleoside methylphosphonates with a chain length of up to nine nucleotidyl units are taken up intact by mammalian cells in culture. All the oligomer analogues tested inhibited, to various extents, colony formation by bacterial, hamster, and human tumor cells in culture.

Nonionic oligonucleotide analogues have been shown to be useful nucleic acid analogues for probing nucleic acid sequence–function relationships both in biochemical experiments and in living cells. Previous reports from this laboratory have described the interaction of nonionic, oligonucleotide ethyl phosphotriesters with transfer RNA (Miller et al., 1974) and the effects of these analogues on cell-free aminoacylation of tRNA (Barrett et al., 1974). A trinucleotide analogue, $G^m_p(Et)G^m_p(Et)U$[1] was shown to be taken up by mammalian cells in culture and to have specific inhibitory effects on cellular protein synthesis and cell growth (Miller et al., 1977).

Recently we described the syntheses of a series of novel nonionic oligonucleotide analogues, the dideoxyribonucleoside methylphosphonates (Miller et al., 1979). These analogues have an isosteric, 3′–5′-linked methylphosphonate group which replaces the normal phosphodiester linkage of nucleic acids. Extensive physical studies by ultraviolet, circular dichroism, and nuclear magnetic resonance spectroscopic techniques revealed that the conformation of these analogues is similar to those of the corresponding phosphodiesters and that the analogues form stable complexes with complementary polynucleotides (Miller et al., 1979; Kan et al., 1980). Since these phosphonate analogues can penetrate mammalian cells and the methylphosphonate linkage is resistant to cleavage by a variety of nucleases, it is of interest to determine if these analogues could be used as probes of the sequence–function relation of nucleic acids in living cells. In this paper we report the synthesis of a series of oligonucleoside methylphosphonates whose base sequences are complementary to the anticodon loops of $tRNA^{Lys}$ species and to the –ACCA–OH amino acid accepting stem of tRNA. The effects of these analogues on cell-free aminoacylation and cell-free protein synthesis were studied. The uptake of selected analogues by mammalian cells in culture and the effects of these compounds on bacterial and mammalian cell growth are reported.

Experimental Section

Materials. Nucleosides were purchased from P-L Biochemicals and were checked for purity by paper chromatography before use. *N*-Benzoyldeoxyadenosine, *N*-isobutyryldeoxyguanosine, their 5′-*O*-dimethoxytrityl derivatives, and 5′-*O*-(methoxytrityl)thymidine were prepared according to published procedures (Schaller et al., 1963; Büchi & Khorana, 1972). d-[(MeO)$_2$Tr]bzA*p*bzA*p*CNEt, d-[(MeO)$_2$Tr]bzA*p*bzAOAc, d-[(MeO)Tr]T*p*T*p*CNEt, d-A*p*T, d-A*p*[illegible], d-T*p*T, and d-T*p*[^{3}H]T were synthesized by procedures previously described (Miller et al., 1979). Dimethyl methylphosphonate (K & K Laboratories) and benzenesulfonic acid (Eastman) were used without further purification. Hydracrylonitrile (Eastman) was dried over 4-Å molecular sieves. Methylphosphonic acid dipyridinium salt and mesitylenesulfonyl tetrazolide were prepared as previously described (Miller et al., 1979). Anhydrous pyridine was prepared by refluxing reagent-grade pyridine (3 L) with chlorosulfonic acid ([illegible] mL) for 7 h, followed by distillation onto sodium hydroxide pellets (40 g). After being refluxed for 7 h, the pyridine was distilled onto 4-Å molecular sieves and stored in the dark.

Silica gel column chromatography was carried out by using Baker 3405 silica gel (60–200 mesh). Thin-layer silica gel chromatography (TLC) was performed on E. Merck silica gel 60 F_{254} plastic-backed TLC sheets (0.2 mm thick). High-pressure liquid chromatography (LC) was carried out by using a Laboratory Data Control instrument on columns (2.1 mm × 1 m) packed with HC Pellosil (Whatman, Inc.). The columns were eluted with a linear gradient (40 mL total) of chloroform to 20% (v/v) methanol in chloroform at a flow rate [illegible]

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[1] Abbreviations used: Np(Et)N, an oligonucleotide ethyl phosphotriester; d-N*p*N*p*N, oligodeoxyribonucleotide analogues containing 3′–5′ internucleoside methylphosphonate linkages (in this abbreviation an italic p represents the methylphosphonate linkage); MST, mesitylenesulfonyl tetrazolide. The symbols used to represent protected nucleosides and oligonucleoside methylphosphonates follow the IUPAC–IUB Commission on Biochemical Nomenclature (1970) recommendations.

of 1 mL/min. Ultraviolet spectra were recorded on a Cary 14 or a Varian 219 ultraviolet spectrophotometer with a temperature-controlled cell compartment. The following extinction coefficients (260 nm) were used: dT, 9100; d-[(MeO)Tr]T, 10 200; d-[$(MeO)_2$Tr]bzA, 12 500; d-bzA, 10 600; d-[$(MeO)_2$Tr]ibuG, 17 400; d-ibuG, 16 700. Paper chromatography was carried out on Whatman 3 MM paper using solvent A: 2-propanol–concentrated ammonium hydroxide–water (7:1:2 v/v).

Preparation of d-[$(MeO)_2$Tr]ibuGpCNEt. d-[$(MeO)_2$Tr]ibuG (12 g, 18.7 mmol) and the pyridinium salt of methylphosphonic acid (21 mmol) were dried by evaporation with anhydrous pyridine (4 × 20 mL) and the residue in 40 mL of pyridine was treated with 2,4,6-triisopropylbenzenesulfonyl chloride (12.7 g, 42 mmol) for 8 h at room temperature. Hydracrylonitrile (4.5 g, 63 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (0.61 g, 2 mmol) were added and the reaction mixture was kept at room temperature. After 2 days the reaction mixture was poured into 500 mL of ice-cold 5% $NaHCO_3$ solution. The solution was extracted with ethyl acetate (2 × 250 mL) and the combined extracts were dried over anhydrous Na_2SO_4. Examination of the extract by TLC showed the presence of both d-[$(MeO)_2$Tr]ibuG*p*CNEt (R_f 0.31, silica gel TLC, 10% MeOH–$CHCl_3$) and d-ibuG*p*CNEt (R_f 0.14, silica gel TLC, 10% MeOH–$CHCl_3$). After concentration the ethyl acetate extract was chromatographed on silica gel (4 × 35 cm) by using ether (1 L) and a 0–20% linear gradient of methanol in chloroform (1.6 L total) as solvents. d-[$(MeO)_2$Tr]ibuG*p*CNEt (2.75 mmol) was obtained in 15% yield while d-ibuG*p*CNEt (2.46 mmol) was obtained in 13% yield. Additional d-[$(MeO)_2$Tr]ibuG*p* (3.69 mmol, 20%) was obtained from the aqueous bicarbonate solution after extraction with chloroform (2 × 200 mL).

Preparation of Protected Oligonucleoside Methylphosphonates. The same general procedures were used as previously described for the preparation of dinucleoside methylphosphonates (Miller et al., 1979). The specific conditions used in the condensation reactions and the yields obtained after silica gel column chromatography are given in Table I. The ultraviolet spectroscopic characteristics and the mobilities of the protected oligonucleotides on silica gel TLC and silica gel high-pressure LC are given in Table II.

Preparation of Oligonucleoside Methylphosphonates. The protecting groups were removed from the blocked oligonucleoside methylphosphonates by using conditions described previously (Miller et al., 1979). In the case of the dA-containing oligomers, the N-benzoyl groups were removed by treatment with hydrazine (Miller et al., 1979). The oligomers were purified by preparative paper chromatography using solvent A. For the ^{3}H-labeled oligothymidine methylphosphonates, d-$(Tp)_n$[^{3}H]T, the condensation reactions containing d-[(MeO)Tr]$(Tp)_n$ plus [^{3}H]TOAc were run on 0.01 (n = 1) and 0.005 (n = 4 and 8) mmol scales while d-G*p*G*p*[^{3}H]T was prepared on a 0.012-mmol scale. The protecting groups were removed without isolation of the protected ^{3}H-labeled oligomers and the entire reaction mixture was chromatographed on paper. The oligonucleoside methylphosphonates were eluted from the paper with 50% aqueous ethanol. The ethanol solutions were passed through DEAE-cellulose columns (0.5 × 1 cm) and stored at 0 °C. The following overall yields were obtained: d-$(Tp)_n$[^{3}H]T (n = 1, 41%; n = 4, 22%; n = 8, 17%) and d-G*p*G*p*[^{3}H]T (15%). The UV spectral properties and chromatographic mobilities of the oligonucleoside methylphosphonates are given in Table III. For use in the physical, biochemical, and biological experiments described below, aliquots containing the required amount of oligomer were evaporated to dryness, and the oligomer was dissolved in the buffer used in the particular experiment.

Interaction of Oligodeoxyadenylate Methylphosphonates with Polynucleotides. The continuous variation experiments and melting experiments were carried out as previously described (Miller et al., 1971). The extinction coefficients of the oligomers were determined by comparing the absorption of a solution of the oligomer in water at pH 7.0 to the absorption of the same solution at pH 1.0. The oligomer extinction coefficient was calculated from the observed hyperchromicity of the oligomer at pH 1.0 by using the following extinction coefficients: dA, pH 1.0, 14.1 × 10^3; dG, pH 1.0, 12.3 × 10^3. The molar extinction coefficient of poly(U) is 9.2 × 10^3 (265 nm) and of poly(dT) is 8.52 × 10^3 (264 nm).

Cell-Free Aminoacylation. (*1*) *E. coli System.* Unfractionated *Escherichia coli* tRNA was purchased from Schwarz/Mann and unfractionated *E. coli* aminoacyl synthetase was purchased from Miles Laboratories, Inc. Reactions were run in 60 μL of buffer containing 100 mM Tris-HCl, pH 7.4, 10 mM $Mg(OAc)_2$, 5 mM KCl, 2 mM ATP, 4 μM ^{3}H-labeled amino acid, 1.8 μM $tRNA_{E.coli}$, and 0–100 μM oligonucleotide, following the procedure of Barrett et al. (1974). Reactions were initiated by addition of 4 μg of aminoacyl synthetase. Aliquots (10 μL) were removed at various times and added to 1 mL cold 10% trichloroacetic acid and the resulting precipitate was filtered on Whatman G/F filters. After being washed with four (1 mL) portions of 2 N HCl and four (1 mL) portions of 95% ETOH, the filters were dried and counted in 7 mL of New England Nuclear 949 scintillation mixture.

(*2*) *Rabbit Reticulocyte System.* A rabbit reticulocyte cell-free translation system was purchased from New England Nuclear (lot no. J1157AW). Reactions were run in 12.5 μL of buffer containing 1 μL of the translation mixture, 79 mM potassium acetate, 0.6 mM magnesium acetate, 57 μM [^{3}H]lysine, and 50 μM oligomer. The reactions wre initiated by addition of 5 μL of reticulocyte lysate and were assayed as described for the *E. coli* system.

Cell-Free Protein Synthesis. (*1*) *E. coli System.* A cell-free protein synthesizing system was isolated from *E. coli* B cells (S-30) according to the procedure of Nirenberg (1963). The system incorporates 300 pmol of [^{3}H]phenylalanine/mg of S-30 protein after 15-min incubation at 37 °C when poly(U) is used as a message.

(*2*) *Rabbit Reticulocyte.* The reticulocyte translation system prepared by New England Nuclear was used. For the translation of globin mRNA, the reactions were run in 12.5 μL of buffer containing 1 μL of the translation mixture, 0.10 μg of globin mRNA (Miles Laboratories), 79 mM potassium acetate, 0.2 mM magnesium acetate, 0–50 μM oligomer, and 20.5 μM [^{3}H]leucine. For the translation of poly(U), the reactions were run in 12.5 μL of buffer containing 1 μL of the translation mixture, 120 mM potassium acetate, 0.8 mM magnesium acetate, 367 μM poly(U), 0–200 μM oligomer (base concentration), and 32 μM [^{3}H]phenylalanine. Reactions were initiated by addition of 5 μL of reticulocyte lysate. Aliquots (2 μL) were removed at various times and added to 1.0 mL of bovine serum albumin (100 μg) solution. The protein was precipitated by heating with 1 mL of 10% trichloroacetic acid at 70 °C, filtered on G/F filters, and counted in 7 mL of Betafluor.

Uptake of Oligodeoxyribonucleoside Methylphosphonates. The uptake of d-A*p*[^{3}H]T, d-G*p*G*p*[^{3}H]T, and d-$(Tp)_n$[^{3}H]T

Table I: Preparation of Protected Oligodeoxyribonucleoside Methylphosphonates

3'-methylphosphonate component (mmol)	5'-OH component (mmol)	MST (mmol)	product (mmol)	yield (%)
d-[(MeO)$_2$Tr]ibuG*p* (0.50)	d-ibuG*p*CNEt (0.50)	2.0	d-[(MeO)$_2$Tr]ibuG*p*ibuG*p*CNEt (0.82)	16
d-[(MeO)$_2$Tr]ibuG*p* (1.0)	d-bzAOAc (1.5)	4.0	d-[(MeO)$_2$Tr]ibuG*p*bzAOAc (0.42)	42
d-[(MeO)Tr]T*p*T*p* (0.33)	d-T*p*T*p*CNEt (0.50)	1.6	d-[(MeO)Tr]T*p*T*p*T*p*T*p*CNEt (0.168)	50
d-[(MeO)Tr]T*p*(T*p*)$_2$T*p*CNEt (0.0324)	d-T*p*(T*p*)$_2$T*p*CNEt (0.0524)	0.16	d-[(MeO)Tr]T*p*(T*p*)$_6$T*p*CNEt (0.0138)	43
d-[(MeO)$_2$Tr]ibuG*p*ibuG*p* (0.07)	d-TOAc (0.15)	0.28	d-[(MeO)$_2$Tr]ibuG*p*ibuG*p*TOAc (0.0153)	22
d-[(MeO)$_2$Tr]bzA*p*bzA*p* (0.065)	d-bzAOAc (0.043)	0.163	d-[(MeO)$_2$Tr]bzA*p*bzA*p*bzAOAc (0.023)	53
d-[(MeO)$_2$Tr]bzA*p*bzA*p* (0.13)	d-bzA*p*bzAOAc (0.20)	0.52	d-[(MeO)$_2$Tr]bzA*p*bzA*p*bzA*p*bzAOAc (0.031)	24
d-[(MeO)$_2$Tr]bzA*p*bzA*p* (0.0168)	d-ibuG*p*bzAOAc (0.0168)	0.0735	d-[(MeO)$_2$Tr]A*p*bzA*p*ibuG*p*bzAOAc (0.0029)	17

Table II: Ultraviolet Spectral Properties and Chromatographic Mobilities of Protected Oligodeoxyribonucleoside Methylphosphonates

	UV spectra[a]						silica gel TLC R_f[b] in MeOH–$CHCl_3$				silica gel HPLC[c]
			$\epsilon_{260}/\epsilon_{230}$		$\epsilon_{280}/\epsilon_{260}$						
oligomer	λ_{max} (nm)	λ_{min} (nm)	calcd	obsd	calcd	obsd	5%	10%	15%	20%	retention time (min)
d-[(MeO)Tr]T*p*T*p*T*p*T*p*CNEt	265 235 sh	243	1.34	1.31	1.55	1.64	–	–	0.08	0.29	–
d-[(MeO)Tr]T*p*(T*p*)$_6$T*p*CNEt	265	243	1.75	0.92	1.57	1.56	–	0.00	–	0.13	–
d-[(MeO)$_2$Tr]ibuG*p*ibuG*p*CNEt	238 253 260 280	225 245 256 270	1.19	1.05	1.33	1.32	–	0.16	–	–	19.2
d-[(MeO)$_2$Tr]ibuG*p*bzAOAc	235 278	256	0.82	0.75	0.88	0.87	–	0.29	–	–	12.3
d-ibuG*p*bzAOAc	260 280	239 267	1.63	1.27	0.90	0.90	–	0.18 0.14	–	–	15.5 17.6
d-[(MeO)$_2$Tr]ibuG*p*ibuG*p*TOAc	240 sh 260 275 sh	228	1.34	1.51	1.38	1.45	–	0.18	–	–	16.0
d-[(MeO)$_2$Tr]bzA*p*bzA*p*bzAOAc	234 280	227 255	0.66	0.61	0.59	0.59	–	0.41 0.38	0.55 0.53	–	13.4 14.3
d-[(MeO)$_2$Tr]bzA*p*bzA*p*bzA*p*bzAOAc	233 sh 280	253	0.71	0.60	0.59	0.60	–	–	0.31	–	19.3
d-[(MeO)$_2$Tr]bzA*p*bzA*p*ibuG*p*bzAOAc	235 sh 280	255	0.89	0.74	0.73	0.75	–	0.15	0.44	–	23.8

[a] Measured in 95% EtOH. [b] E. Merck silica gel 60 F_{254} sheets, 0.2 mm thick. [c] HC Pellosil (2.1 mm × 1 m); 0–20% methanol in chloroform; 1 mL/min; 40-mL total volume.

by transformed Syrian hamster fibroblasts was determined as previously described (Miller et al., 1977).

Effects of Oligodeoxyribonucleoside Methylphosphonates on Colony Formation. (*1*) *E. coli*. *E. coli* B was grown in M-9 medium (Bolle et al., 1968) supplemented with glucose (36 g/L) and 1% casamino acids. The cells were harvested in mid-log phase and resuspended in 50 μL of fresh medium containing 0–160 μM oligomer at a final cell density of 1 × 10^4 cells/mL. The cells were incubated for 1 h at 37 °C and then diluted with 0.9 mL of medium. A 0.8-mL aliquot was added to 2.5 mL of 0.8% Bactoagar at 45 °C. This solution was quickly poured onto a 100-mm plate containing solid 1.2% Bactoagar. After solidification, the plates were incubated overnight at 37 °C and the resulting colonies were counted.

(*2*) *Transformed Syrian Hamster Embryonic Fibroblasts (BP-6) and Transformed Human Fibroblasts (HTB1080).* Colony formation by the fibroblasts in the presence of the methylphosphonate analogues was carried out as previously described (Miller et al., 1977).

Results

Synthesis of Oligodeoxyribonucleoside Methylphosphonates. The synthetic scheme used for preparing the oligonucleoside methylphosphonates followed the basic approach used to synthesize dideoxyribonucleoside methylphosphonates (Miller et al., 1979). Suitably protected monomers or oligomer blocks carrying a 3'-terminal methylphosphonate group were condensed with protected mono- or oligonucleotides bearing a free 5'-hydroxyl group. Mesitylenesulfonyl tetrazolide (Stawinsky et al., 1977) was used as the condensing agent. The fully protected oligomers were purified by silica gel column chromatography. The reaction conditions used and the yields obtained are given in Table I. The oligomers were characterized by ultraviolet spectroscopy, thin-layer chromatography, and high-pressure liquid chromatography as indicated in Table II.

The protecting groups were removed as previously described (Miller et al., 1979). In the case of the deoxyadenosine-containing oligomers, the *N*-benzoyl groups were first removed by treatment with hydrazine hydrate (Letsinger et al., 19[illegible]). The remaining 3'-*O*-acetyl and 5'-*O*-dimethoxytrityl groups were removed by sequential treatment with ammonium hydroxide and 80% acetic acid. The oligomers were purified by preparative paper chromatography and were characterized by UV spectroscopy (Table III).

Interaction of Oligodeoxyribonucleoside Methylphosphonates with Complementary Polynucleotides. Table IV summarizes thhe melting temperatures of complexes formed between oligodeoxyadenosine methylphosphonates and poly(U) or poly(dT). For comparison, the melting temperatures of complexes formed by oligodeoxyribo- and oligoribo-adenosines are included. Each oligomer forms a triple-stranded complex with a stoichiometry of 2U:1A or 2T:1A. The melting temperatures increase as the chain length of the oligonucleotide increases. For a given chain length, the complexes formed by the methylphosphonate analgoues melt at higher temperatures than those formed by the natural diester oligomers. With the exception of r-ApApApA, the complexes formed by the oli-

Table III: Spectral Properties and Chromatographic Mobilities of Oligodeoxyribonucleoside Methylphosphonates

oligomer	UV spectra[a] λ_{max} (nm)	λ_{min} (nm)	$\epsilon_{260}/\epsilon_{230}$	ϵ at λ_{max}	paper chromatography[b] R_f, solvent A
d-GpGpT[c]	257 270 sh	230	1.45	33.4×10^3	0.31
d-ApApA	258	232	4.27	39.0×10^3	0.29
d-ApApApA	258	230	3.77	50.4×10^3	0.11
d-ApApGpA	258	227	3.03	50.3×10^3	0.11
d-Tp[^{3}H]T	267	235	1.53		0.59
d-(Tp)$_4$[^{3}H]T	266	235	1.49		0.21
d-(Tp)$_8$[^{3}H]T	266	235	1.56		0.17

[a] Measured in water, pH 7.0. [b] R_f^A of pT = 0.11. [c] The UV spectrum is similar to that of d-GpGpT (Miller et al., 1974).

Table IV: Interaction of Oligonucleoside Methylphosphonates with Complementary Polynucleotides[a]

oligomer	T_m with poly(U) (2U:1A) (°C)	T_m with poly(dT) (2T:1A) (°C)
d-ApA: isomer 1	15.4	18.7
isomer 2	19.8	18.4
d-ApApA	33.0	36.8
d-ApApApA	43.0	44.5
d-ApA	7.0	9.2
d-ApApApA	32.0	35.5
r-ApApApA	36.2	2.4

[a] 5×10^{-5} M total [nucleotide], 10 mM Tris, and 10 mM $MgCl_2$, pH 7.5.

gomers with poly(dT) have slightly higher melting temperatures than the corresponding complexes formed with poly(U).

The interaction of d-GpGp[^{3}H]T with unfractionated tRNA$_{E.coli}$ was measured by equilibrium dialysis (Miller et al., 1974). The apparent association constants at 0, 22, and 37 °C are 1100 M^{-1}, 200 M^{-1}, and 100 M^{-1}, respectively. These binding constants are much lower than those of the 2′-O-methylribooligonucleotide ethyl phosphotriester, G^m_p(Et)-G^m_p(Et)[^{3}H]U, which are 9300 M^{-1} (0 °C), 1900 M^{-1} (22 °C), and 2000 M^{-1} (37 °C) (Miller et al., 1977).

Effect of Oligodeoxyribonucleoside Methylphosphonates on Cell-Free Aminoacylation of tRNA. The effects of selected oligodeoxyribonucleoside methylphosphonates on aminoacylation of unfractionated tRNA$_{E.coli}$ are shown in Table V. Three amino acids were tested at various temperatures. The deoxyadenosine-containing analogues which are complementary to the –UUUU– sequence of the anticodon of tRNA$^{Lys}_{E.coli}$ have the largest inhibitory effect on aminoacylation of tRNA$^{Lys}_{E.coli}$. The percent inhibition increases with increasing chain length and decreases with increasing temperature. Inhibition by d-ApApGpA and by the diesters d-ApApApA and r-ApApApA is less than that exhibited by d-ApApApA. In contrast to their behavior with tRNa$^{Lys}_{E.coli}$, neither the methylphosphonates, d-ApApApA and d-ApApGpA, nor the phosphodiesters, d-ApApApA and r-ApApApA, had any inhibitory effect on tRNA$^{Lys}_{rabbit}$ in the rabbit reticulocyte cell-free system (data not shown).

Effects of Oligodeoxyribonucleoside Methylphosphonates on Cell-Free Protein Synthesis. The ability of deoxyadenosine-containing oligonucleoside methylphosphonates to inhibit polypeptide synthesis in cell-free systems directed by synthetic and natural messages was tested. The results of these experiments are given in Table VI. Poly(U)-directed phenylalanine incorporation and poly(A)-directed lysine incorporation are both inhibited by oligodeoxyadenosine methylphosphonates and diesters in the *E. coli* system at 22 °C. The percent inhibition increases with oligomer chain length and is greater for polyphenylalanine synthesis. The methylphosphonate analogues are more effective inhibitors than either

Table V: Effects of Oligonucleoside Methylphosphonates on Aminoacylation in an *E. coli* Cell-Free System

oligomer[a]	% inhibition[b] Phe, 0 °C	Leu, 0 °C	Lys 0 °C	Lys 22 °C	Lys 37 °C
d-ApA	6	0	7		
d-ApApA	9	0	62	15	0
d-ApApApA	9	12	88	40	16
d-ApApGpA	12	12	35	0	
d-GpGpT	31	5	34	9	15
dGpGpT (400 μM)	23				
d-ApApApA	0	7	71[c]	15[c]	
r-ApApApA			78[d]	17[d]	

[a] [oligomer] = 50 μM. [b] [tRNA$_{E.coli}$] = 2 μM. [c] [oligomer] = 100 μM. [d] [oligomer] = 125 μM.

Table VI: Effects of Oligonucleoside Methylphosphonates on Bacterial and Mammalian Cell-Free Protein Synthesis at 22 °C

oligomer	% inhibition *E. coli* poly(U) directed[a]	*E. coli* poly(A) directed[b]	rabbit reticulocyte poly(U) directed[a]	rabbit reticulocyte globin mRNA directed[c]
d-ApA	20	10		
d-ApApA	84	30	81	
d-ApApApA	100	65	77	0
d-ApApGpA	22			0
d-ApApApA	13	19	18	0
r-ApApApA	18	17	85	0

[a] [poly(U)] = 360 μM in U; [oligomer] = 175–200 μM in base. [b] [poly(A)] = 300 μM in A; [oligomer] = 175–200 μM in base. [c] [oligomer] = 200 μM in base.

d-ApApApA or r-ApApApA at the same concentration. Although both the oligodeoxyadenosine methylphosphonates and the phosphodiesters inhibit translation of poly(U) in the rabbit reticulocyte system, no effect on the translation of globin message was observed. As in the case of the *E. coli* system, inhibition of phenylalanine incorporation increased with oligomer chain length and was greater for the methylphosphonate analogues than for the diesters.

Uptake of Oligodeoxyribonucleoside Methylphosphonates by Mammalian Cells. Figure 1 shows the incorporation of radioactive 100 μM d-GpGp[^{3}H]T with time into transformed Syrian hamster embryonic fibroblasts growing in monolayer. The incorporation is approximately linear for the first hour and begins to level off after 1.5 h. The concentration of radioactivity inside the cells is ~117 μM after 1.5 h assuming a cell volume of 1.5 μL/10^6 cells (Hempling, 1972).

Cells were incubated with 25 μM d-GpGp[^{3}H]T for 18 h. The medium was removed, and the cells were washed with phosphate buffer and then lysed with NaDodSO$_4$. Approx-

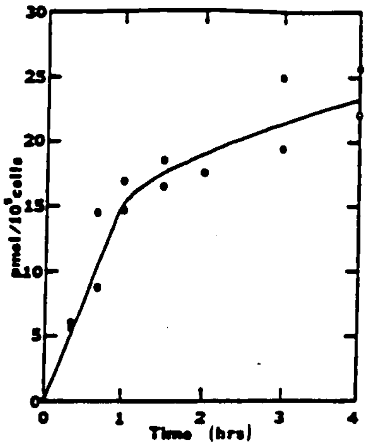

FIGURE 1: Transport of (○) 100 µM d-GpGp[^{3}H]T and (●) 100 µM d-(Tp)$_8$[^{3}H]T into transformed Syrian hamster fibroblasts growing in monolayer at 37 °C.

imately 30% of the total radioactivity from the lysate was found in Cl_3AcOH-precipitable material. The DNA was precipitated from the lysate and digested with deoxyribonuclease and snake venom phosphodiesterase. The culture medium, the DNA-free lysate, and the DNA digest were each examined by paper chromatography. Only intact d-GpGp-[^{3}H]T was found in the medium. Radioactivity corresponding to [^{3}H]TTP (6%) and to d-GpGp[^{3}H]T (94%) was found in the lysate, while the DNA digest gave [^{3}H]dpT and [^{3}H]dT as products.

Similar uptake studies were carried out with d-Ap[^{3}H]T and with a series of oligothymidylate analogues, d-(Tp)$_n$[^{3}H]T (n = 1, 4, and 8). The rates and extents of uptake of these analogues were very similar to that of d-GpGp[^{3}H]T (Figure 1). Examination of the culture medium and cell lysate after overnight incubation with these oligonucleotides gave results similar to those found for d-GpGp[^{3}H]T.

Effects of Oligodeoxyribonucleoside Methylphosphonates on Colony Formation by Bacterial and Mammalian Cells. The effects of selected oligodeoxyribonucleoside methylphosphonates on colony formation by *E. coli* B, transformed Syrian hamster fibroblast (BP-6), and transformed human fibroblast (HTB 1080) cells are summarized in Table VII. The d-(Ap)$_n$A analogues appear to inhibit *E. coli* colony formation at high concentrations (160 µM). However, no inhibitory effects on the incorporation of [^{3}H]leucine into cellular protein or [^{3}H]thymidine into cellular DNA could be detected in the presence of these compounds.

Colony formation of both transformed hamster and human cells are inhibited to various extents by the oligonucleoside methylphosphonates. Both the hamster and human cells appear to be affected to a similar extent by a given analogue. It appears in the case of d-ApA that each diastereoisomer exerts a different inhibitory effect on the growth of the hamster cells. As in the case of *E. coli*, no inhibition of cellular protein synthesis could be detected.

Discussion

Oligodeoxyribonucleoside methylphosphonates with sequences complementary to the anticodon loop of tRNA^{Lys} and to the –ACCA–OH amino acid accepting stem of tRNA were prepared in a manner similar to that used to prepare dideoxyribonucleoside methylphosphonates (Miller et al., 1979). The present studies demonstrate the ability to join blocks of protected methylphosphonates to give oligomers with chain lengths up to nine nucleotidyl units. The yields in these

Table VII: Effects of Oligonucleoside Methylphosphonates on Colony Formation by Bacterial and Mammalian Cells in Culture

	% inhibition[a]			
	E. coli B			
oligomer	50 µM	160 µM	BP-6, 50 µM	HTB 1080, 50 µM
d-ApT	4	5	5, 16[b]	12
d-ApA	8	58	6, <1[b]	5
d-ApApA	3	44	29	31
d-ApApApA	19	78	36	19
d-GpGpT	7	11	7	9

[a] The results are the average of two or three experiments. Each experiment consisted of two plates (bacterial cells) or three plates (mammalian cells). The average variation is ±3% in percent inhibition. The cells were treated with and grown in the presence of the oligomer at 37 °C. [b] The percent inhibition of isomers 1 and 2, respectively.

condensation reactions are acceptable, although reactions involving deoxyguanosine residues appear to proceed in low yield. Similar difficulties have been encountered in the syntheses of oligonucleotide phosphotriesters. Unlike the dideoxyribonucleoside methylphosphonates previously reported, the oligodeoxyribonucleoside methylphosphonates prepared for this study were not resolved into their individual diastereoisomers.

The oligodeoxyadenosine analogues form triple-stranded complexes with both poly(U) and poly(dT). These complexes are more stable than similar complexes formed by either oligoribo- or oligodeoxyribonucleotides. As previously suggested for oligonucleotide ethyl phosphotriesters (Miller et al., 1971, 1974; Pless & Ts'o, 1977) and dideoxyribonucleoside methylphosphonates (Miller et al., 1979), this increased stability is attributed to the decreased charge repulsion between the nonionic backbone of the analogue and the negatively charged complementary polynucleotide backbone. With the exception of r-ApApApA (Table IV), the stability of the complexes formed with poly(dT) are slightly higher than those formed with poly(U), a situation which is also observed for the interaction of poly(dA) with poly(dT) and with poly(U) (Chamberlin, 1965). The lower stability of the (r-ApApApA)·2[poly(dT)] complex is also reflected at the polymer level. Thus, under the conditions of the experiments described in Table IV, we found that the T_m of poly(rA)·2[poly(rU)] is 83 °C while the T_m of poly(rA)·2[poly(dT)] is 59 °C. These results are consistent with those of Riley et al. (1966). They observed that formation of the poly(rA)·2[poly(dT)] complex occurs only at a sodium ion concentration of 2.5 M in the absence of magnesium, while poly(rA)·2[poly(rU)] forms in 0.1 M sodium phosphate buffer.

The oligodeoxyadenosine methylphosphonates and their parent diesters selectively inhibit cell-free aminoacylation of tRNA$^{Lys}_{E.coli}$. The extent of inhibition is temperature dependent and parallels the ability of the oligomers to bind to poly(U). These observations and the previously demonstrated interaction of r-ApApApA with tRNA$^{Lys}_{E.coli}$ (Möller et al., 1978) suggest that inhibition occurs as a result of oligomer binding to the –UUUU– anticodon loop of the tRNA. The reduced inhibition observed with d-ApApGpA is consistent with this explanation, since interaction of this oligomer with the anticodon loop would involve formation of a less stable G·U base pair.

Recent studies by Ramberg et al. (1978) have shown that the rate of aminoacylation of tRNA$^{Lys}_{E.coli}$ substituted with 5-fluorouracil is considerably lower than that of nonsubstituted tRNA$^{Lys}_{E.coli}$. The increased K_m of the 5-fluorouracil-substituted tRNA suggested a decreased interaction with the lysyl aminoacyl synthetase. These results and those of Saneyoshi

& Nishimura (1971) suggest that the anticodon loop of $tRNA^{Lys}_{E.coli}$ is part of the synthetase recognition site. Thus, inhibition of aminoacylation by the oligodeoxyribonucleoside methylphosphonates could result from the reduction in the affinity of the synthetase for $tRNA^{Lys}$–oligonucleotide complexes. The greater inhibition observed with d-A*p*A*p*A*p*A vs. the diesters, d-ApApApA or r-ApApApA, may result from greater binding of the analogue to the anticodon loop or to the decreased ability of the synthetase to displace the nonionic oligonucleotide analogue vs. the phosphodiester oligomers from the anticodon loop. Alternatively, oligomer binding to the anticodon loop could induce a conformational change in the tRNA, leading to a lower rate and extent of aminoacylation. Such conformational changes have been detected when r-ApApApA binds to $tRNA^{Lys}_{E.coli}$ (Möller et al., 1979; Wagner & Garrett, 1979).

None of the oligomers have any effect on the aminoacylation of $tRNA^{Lys}_{rabbit}$ in a cell-free system. Since the anticodon regions of tRNAs from bacterial and mammalian sources probably are similar, the oligo(A) analogues are expected to interact with the anticodon region of both $tRNA^{Lys}$'s. The failure to observe inhibition of aminoacylation of $tRNA^{Lys}_{rabbit}$ in the presence of these oligo(d-A) analogues suggests that there may be a difference between the interaction of the lysine aminoacyl synthetase with $tRNA^{Lys}$ from *E. coli* and from rabbit systems or a difference between the structure of these two $tRNA^{Lys}$'s in response to the binding of oligo(d-A) analgoues.

The trimer, dG*p*G*p*T, inhibits both phenylalanine and lysine aminoacylation at 0 °C but has little effect on leucine aminoacylation. The aminoacyl stems of both $tRNA^{Lys}_{E.coli}$ and $tRNA^{Phe}_{E.coli}$ terminate in a G-C base pair between nucleotides 1 and 72, while a less stable G-U base pair is found at this position in $tRNA^{Leu}_{E.coli}$ (Sprinzl et al., 1978). Thus the observed differences in inhibition of aminoacylation by d-G*p*G*p*T may reflect differences in the ability of this oligomer to bind to the different –ACC– ends of the various tRNAs.

Inhibition of lysine aminoacylation by dG*p*G*p*T is very temperature sensitive and parallels the decrease in binding to tRNA with increasing temperature. This behavior of d-G*p*G*p*T contrasts that of $G^m_p(Et)G^m_p(Et)U$ (Miller et al., 1977). Although both oligomers can potentially interact with the same sequences in tRNA, the 2'-*O*-methylribotrinucleotide ethyl phosphotriester binds more strongly and more effectively inhibits aminoacylation. The differences in binding ability may be due to overall differences in the conformation of the deoxyribo vs. 2'-*O*-methylribo backbones of these oligomers.

The oligodeoxyribonucleoside methylphosphonates effectively inhibit polyphenylalanine synthesis in cell-free systems derived from both *E. coli* and rabbit reticulocytes. In the *E. coli* system, the extent of inhibition by the oligodeoxyadenosine analogues parallels the T_m values of the oligomers with poly(U). The tetramer, d-A*p*A*p*G*p*A, which would have to form a G·U base pair with poly(U), was 4.5-fold less effective than d-A*p*A*p*A*p*A. These results suggest that the oligomers inhibit polypeptide synthesis as a consequence of forming complexes with the poly(U) message. A similar inhibitory effect by poly(dA) on the translation of poly(U) was observed by Williamson et al. (1967). It is unlikely that inhibition results from nonspecific interaction of the methylphosphonates with protein components of the translation systems. In the *E. coli* system, poly(A) translation is inhibited to a lesser extent than is translation of poly(U), while in the reticulocyte system, no inhibition of globin mRNA translation is observed.

The data suggest that the magnitude of inhibition of poly(U)-directed polypeptide synthesis in the *E. coli* system does not reflect proportionally the ability of the oligomer to bind to poly(U). Although the oligomer pairs d-A*p*A*p*A/d-ApApA and d-A*p*A*p*A*p*A/r-ApApApA form complexes with poly(U) which have very similar T_m values (see Table IV), in each case the methylphosphonate analogues inhibit 5.5–6.5 times better than do the diesters. This stronger inhibitory effect could result from a decreased ability of the ribosome to displace the nonionic oligodeoxyribonucleoside methylphosphonates from the poly(U) meassage, or, alternatively, there may be a degradation of the oligonucleotides (phosphodiesters) by nucleases in the cell-free translation systems but not the corresponding phosphonate analogues.

Experiments with radioactively labeled oligonucleotide methylphosphonates show that these analogues are taken up by mammalian cells growing in culture. The extent of uptake is consistent with passive diffusion of the oligomer across the cell membrane. Both d-T*p*[^{3}H]T and d-(T*p*)$_8$[^{3}H]T are taken up to approximately the same extent, which suggests that there is no size restriction to uptake over this chain length range. This behavior is in contrast to results obtained with *E. coli* B cells (K. Jayaraman et al., unpublished results).

Examination of lysates of mammalian cells exposed to labeled oligomers for 18 h showed that ~70% of the labeled thymidine was associated with intact oligomer with the remainder found in thymidine triphosphate and in cellular DNA. These observations indicate that the oligodeoxyribonucleoside methylphosphonates, which are recovered intact from the culture medium, are slowly degraded within the cell. Failure to observe shorter oligonucleotides and the known resistance of the methylphosphonate linkage to nuclease hydrolysis suggests that degradation may result from cleavage of the 3'-terminal [^{3}H]thymidine *N*-glycosyl bond with subsequent reutilization of the thymine base.

The uptake process of the oligonucleoside methylphosphonates is quite different from that of previously studied oligonucleotide ethyl phosphotriesters (Miller et al., 1977; P. S. Miller et al., unpublished results). In the case of $G^m_p(Et)G^m_p(Et)[^3H]U$, the oligomer is rapidly taken up by the cells and is subsequently deethylated. Further degradation to smaller oligomers is then observed, presumably as a result of nuclease-catalyzed hydrolysis of the resulting phosphodiester linkages. Approximately 80% of the oligomer is metabolized within 24 h. Although the rate of uptake of d-Gp(Et)Gp(Et)[^{3}H]T is similar to that of d-G*p*G*p*[^{3}H]T, examination of the cell lysate showed extensive degradation of the phosphotriester analogue. The relatively long half-lives of the oligodeoxyribonucleoside methylphosphonates may be of value in potential pharmacological applications of these oligonucleotide analogues.

The effects of these analogues on cell colony formation confirmed that the methylphosphonates are taken up by both mammalian and bacterial cells. All the oligomers tested inhibited colony formation of both cell types to various extents. The mechanism(s) by which these compounds exert their inhibitory effects is (are) currently under investigation. No decrease in either overall short-term cellular protein synthesis or DNA synthesis was detected by the present procedure in the presence of these compounds. This does not rule out the possibility that the syntheses of certain critical proteins are perturbed by these oligomers. We are currently studying this possibility by examination of the cellular proteins using two-dimensional gel electrophoresis.

The experiments described in this paper extend our studies on the use on nonionic oligonucleotides as sequence–function probes of nucleic acids both in biochemical experiments and

in living cells. In a future publication we will describe the effects of an oligodeoxyribonucleoside methylphosphonate complementary to the 3′ terminus of 16S rRNA on bacterial protein synthesis and growth (K. Jayaraman et al., unpublished results). Our results suggest that sequence-specific oligonucleoside methylphosphonates may find important applications in probing and regulating nucleic acid function within living cells.

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PHOSPHORUS CHEMISTRY DIRECTED TOWARDS BIOLOGY

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF SOME ANALOGUES OF NUCLEIC ACIDS COMPONENTS

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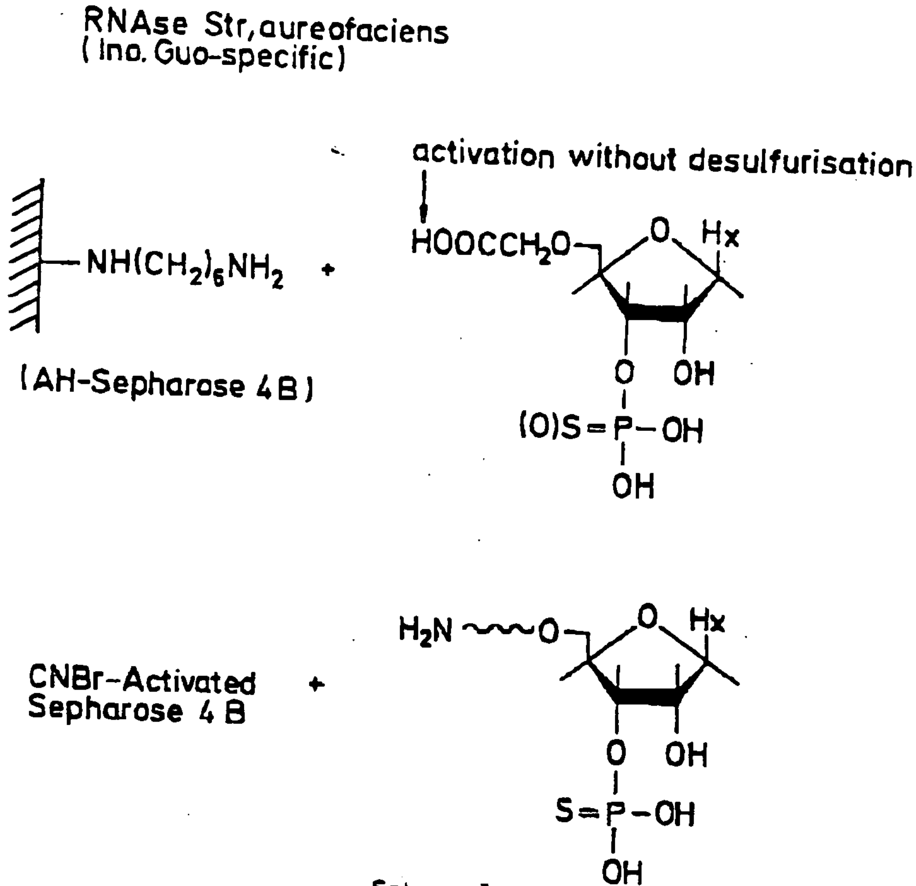
Abstract - Three novel types of nucleotide analogues have been synthetized and investigated in vitro, namely, (a) inosine 2´(3´)-phosphate derivatives containing 5´-O-linking group for the binding to Sepharose supports, (b) 5´-O-phosphorylmethyl derivatives of ribonucleotides which are effective competitive inhibitors of some 5´-nucleotidases and (c) phosphomonoester and phosphodiester derivatives of four 9-(2,3,4-trihydroxybutyl)adenines; ribonuclease T2 cleaves 2,3-cyclic phosphodiesters with absolute configuration 2S.

Nucleotide analogues modified both in the alcoholic part of the molecule and in the phosphorus moiety are useful tools for investigation and isolation of enzymes connected with nucleic acids metabolism. The present contribution describes three novel types of such analogues and their possible application for the above purposes.

1. INOSINE 2´(3´)-THIOPHOSPHATE DERIVATIVES FOR AFFINITY CHROMATOGRAPHY

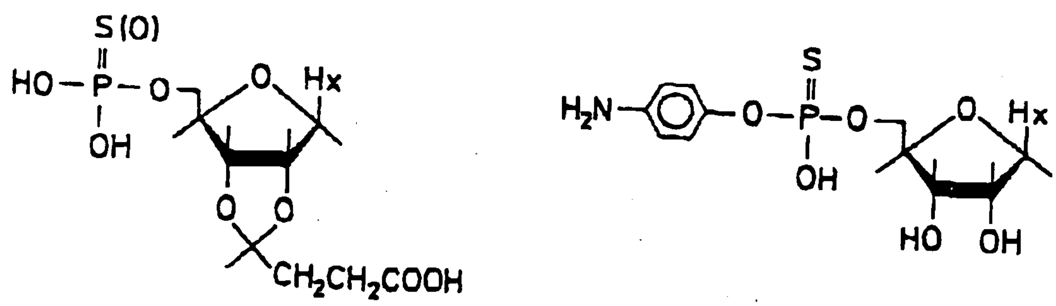
Affinity chromatography is regarded as one of the most efficient methods for the purification of enzymes. Its principle consists in binding of a compound with an increased affinity towards the particular enzyme, to an insoluble support. On the basis of an intermolecular interaction, the protein is tightly bound or retarded on the support column and, after the removal of contaminants, is eluted under the conditions which dissociate the complexes involved (increased ionic strength, pH etc.). For nucleases, satisfactory results have been obtained with thiophosphate analogues as active ligands (1,2). The binding of an analogue to a polymer support requires the presence of an additional grouping of a sufficient length mediating the covalent link; the reaction leading to such a linkage must not affect the inhibitory activity of the ligand, or the properties of the support either.

In our particular case we have been dealing with ribonuclease Streptomyces aureofaciens which is known to split specifically the ester bonds of 3´-guanylic and 3´-inosinic acid (3,4). The synthesis of possible inhibitors was therefore undertaken in the series of inosine derivatives only. It was possible to predict that the 2´(3´)-thiophosphates of this series might exhibit inhibitory activity, suitable for affinity chromatography. Keeping the hypoxanthine moiety intact (preliminary studies have shown that any change of this part results in loss of affinity) (4), the only other position left for the link to the support is the 5´-position. We have chosen 5´-O-carboxymethylinosine derivatives as compounds with a stable (ether) bond to the nucleoside, which offers various methods for binding to the support. Two different types of binding have been employed: (a) binding of an activated carboxymethyl group to the 6-aminohexyl-Sepharose 4A and (b) binding of an aminoalkylamide of the former nucleoside derivative to the cyanogen-bromide-activated Sepharose 4A (Scheme 1). Both types do not practically differ in the character of the link, but they do differ in the length of the spacer group. In the both series, the ligands tested contained both 2´(3´)-phosphate groups and/or thiophosphate groups.



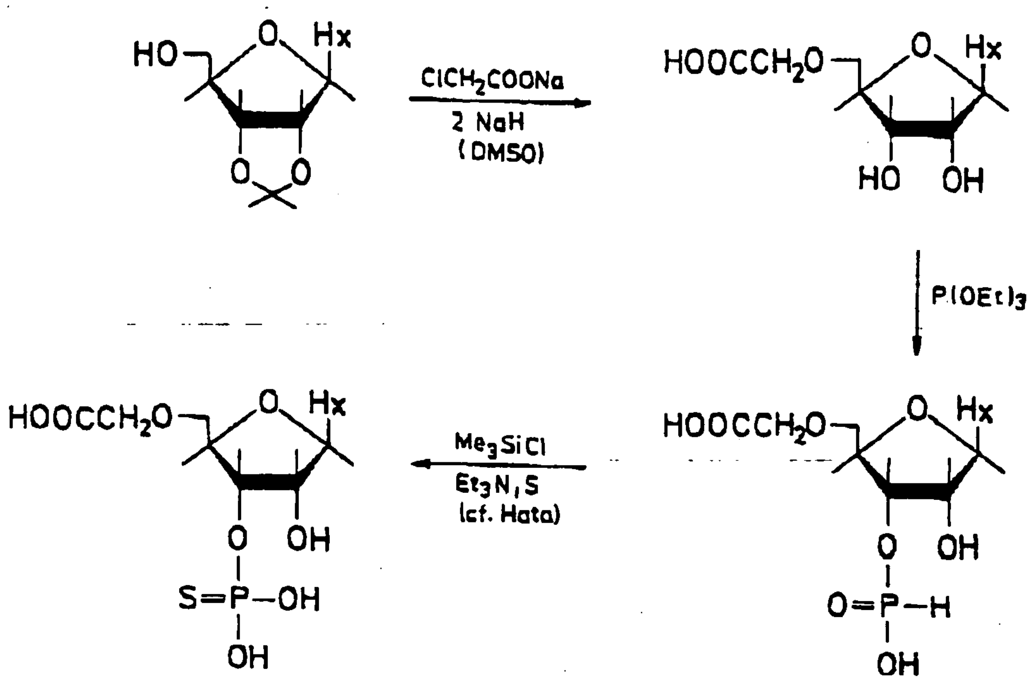
Scheme 1

For comparatory purposes, the derivatives of inosine 5′-phosphate and 5′-thiophosphate linked via the levulinate spacer group (5) were also prepared by the reaction of the corresponding 5′-nucleotides with ethyl orthoformate and ethyl levulinate followed by alkaline hydrolysis (Scheme 2).



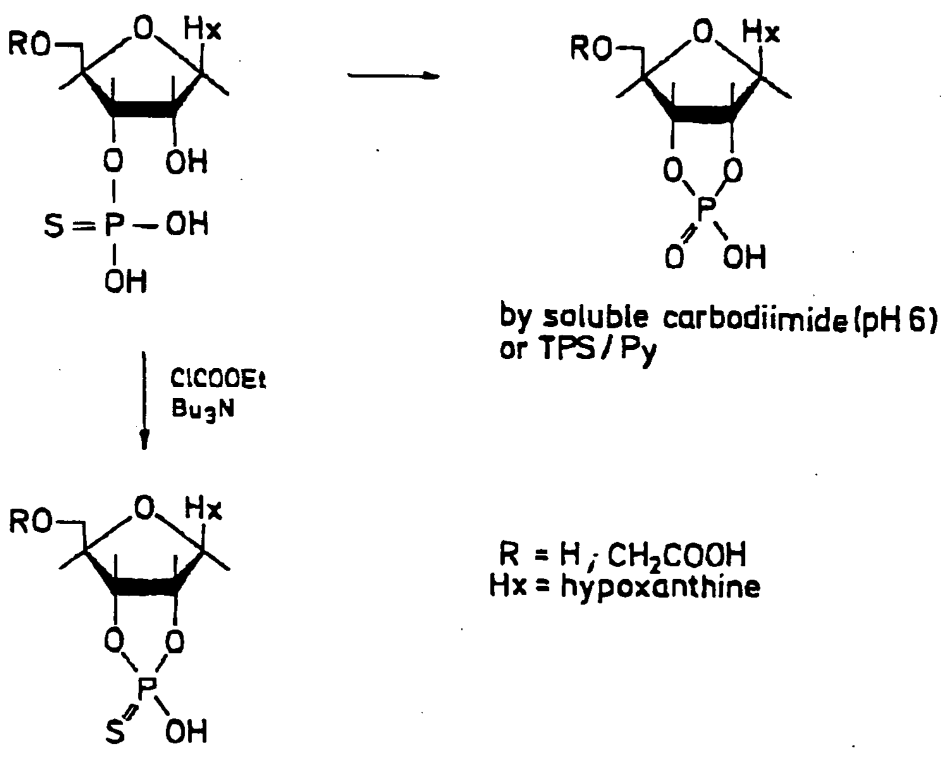
Scheme 2

5′-O-Carboxymethylinosine was prepared by the reaction of 2′,3′-protected inosine with sodium chloroacetate in the presence of sodium hydride (cf.6). The optimum conditions involve the use of two molar equivalents of sodium hydride and dimethyl sulfoxide as a solvent. After removal of the protecting group, the material obtained was purified by anion exchange chromatography. The formation of the N(1)-substituted derivative and/or the 5′,N(1)-disubstituted derivative was minimal under the conditions employed. The 5-O-carboxymethyl derivative was transformed to a 2′(3′)-phosphite by acid catalyzed transesterification with triethyl phosphite (7) and then to the 2′(3′)-thiophosphate by the elegant method of Hata (8), i.e. reaction with trimethylsilyl chloride and sulfur (Scheme 3).



Hx = hypoxanthine

Scheme 3

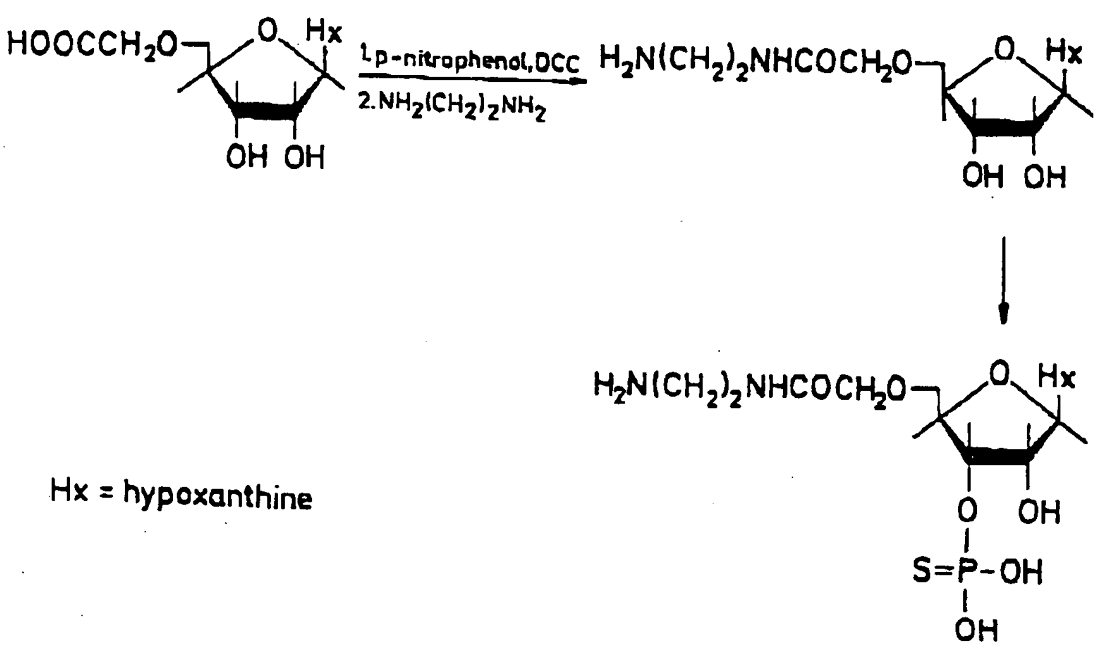


Scheme 4

The binding of an analogue bearing the 5'-O-carboxymethyl function towards aminohexyl-Sepharose requires an activation of the carboxyl group. However, this process is unequivocally accompanied by a similar reaction at the thiophosphate moiety. Consequently, due to the presence of neighbouring hydroxyl group at the sugar moiety, the sulfur is easily eliminated under the 2',3'-cyclic phosphate formation. With water-soluble carbodiimides, this reaction is complete within few minutes at pH 6-7, equally with N,N-dicyclohexylcarbodiimide or, contrary to the previous observation (9), also with 2,4,6-triisopropylbenzenesulfonylchloride in anhydrous medium. These reactions were checked with inosine 2'(3')-thiophosphate as a model compound (Scheme 4). Surprizingly, it was found that the mixed-anhydride method with ethyl chloroformate (10) is the procedure of choice which gives the 2',3'-cyclic thiophosphate without appreciable loss of sulfur (monitored by HPLC); the compound mentioned is, unlike the other guanyl-specific ribonuclease T1 (cf.11), quite resistant to the action of the ribonuclease Streptomyces aureofaciens.

The binding of 5'-O-carboxymethyl derivatives of nucleosides to proteins by the chloroformate procedure has been already described in previous papers (12,13) and is therefore applicable also for AH-Sepharose and the above thiophosphate analogue, though, at the same time, cyclisation probably occurs.

The alternative approach (Scheme 5) starts again with the 5'-O-carboxymethyl derivative which is converted to the 2-aminoethylamide via the p-nitrophenyl ester (cf.14); the separation of the product from the accompanying N,N'-disubstituted amide proceeds easily by cation exchange chromatography. The transformation to the 2'(3')-phosphite and, subsequently, the 2'(3')-thiophosphate occurs similarly as in the Scheme 4. The final product is ready for binding to the cyanogen-bromide-activated Sepharose.



Scheme 5

All the materials prepared have been linked to the corresponding support materials by the usual procedures and their affinity towards the enzyme was tested by both static experiments and column runs. The results obtained are qualitatively summarized in Table 1.

From these data it follows that the ligands containing 2'(3')-thiophosphate are more effective than 5'-thiophosphate derivatives, both of them being more active than the phosphate containing molecules. It seems that the most active material is the AH-Sepharose loaded by 5'-O-carboxymethyl-inosine 2'(3')-thiophosphate (or 2',3'-cyclothiophosphate). At the same time, the differences mentioned confirm that the affinity cannot be due to the ion exchanger character of the modified Sepharose. However, since the additional factors (retardation of inactive proteins, recovery of enzyme and/or release of contaminating pigments) are of importance in the practical evaluation, the final choice of the best material will await further investigation.

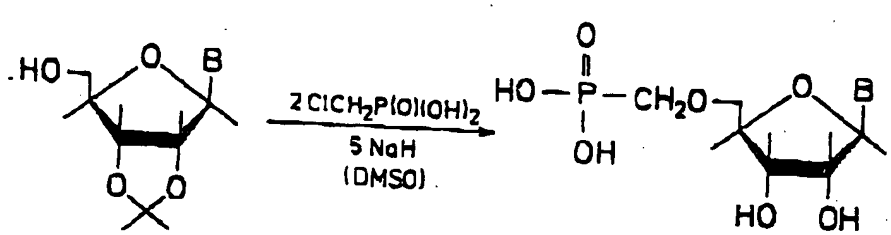
Table 1. Affinity binding of Str.aureofaciens RNAse to the modified Sepharose 4B (pH 6.5-8.0)

Ligand	Sepharose 4B	Binding
5'-CM-Ino-p_s	AH	strong
5'-CM-Ino-p^s	AH	medium
5'-AECM-Ino-p_s	BrCN	strong
2',3'-Lev-IMP^s	AH	medium
2,3'-Lev-IMP_s	AH	medium
IMP_s-(p-aminophenyl)	BrCN	weak

Abbreviations: p_s... 2'(3')-thiophosphate, p... 2'(3')-phosphate, CM... carboxymethyl, AECM... 2-aminoethylaminocarbonylmethyl, Lev... levulinyl, IMP_s... inosine 5-thiophosphate

2. 5'-O-PHOSPHONYLMETHYL DERIVATIVES OF RIBONUCLEOTIDES-A NOVEL TYPE OF 5'-NUCLEOTIDE ANALOGUES

The idea of nucleotide analogues resistant towards the action of dephosphorylating enzymes was followed for many years in various laboratories. The compounds investigated include nucleoside phosphites (7,15-17), alkanephosphonates (18-21), hydroxy-(22) and aminoalkanephosphonates (23) 5'-deoxynucleoside phosphonates (24-29) and the already mentioned thiophosphate analogues (9,11,30-34). Many of these compounds also exhibit significant inhibitory activity towards various enzymes. In the course of our work we have been stimulated by the finding (35) that the response of phosphomonoesterases can be to a large extent affected by the presence or absence of an oxygen atom in the vicinity of phosphorus thus causing a decrease in inhibitory activity between the alkyl(aryl)phosphates and phosphonates. Therefore, we undertook the synthesis of novel nucleotide analogues derived from hydroxymethanephosphonic acid containing the nucleoside moiety bound by a (comparatively) stable ether linkage to the hydroxymethyl group. The reaction scheme for the synthesis (Scheme 6) of the 5'-isomers consists in treatment of ribonucleoside 2',3'-isopropylidene derivatives with disodium salt of chloromethanephosphonic acid. The reaction is best performed with sodium hydride in dimethyl sulfoxide; the compounds containing acid NH-linkage (uracil, hypoxanthine) require excess of sodium hydride to generate the nucleophilic anion of the 5'-hydroxyl group. After removal of the protecting group at pH 2, the products are isolated by the usual ion exchange techniques. The substitution at 5' can be achieved also with cytidine and adenosine derivatives with or without protection of the exo-amino function; however, due to the simultaneous substitution at N(3) or N(1), respectively, some losses of the required products are at hand.



B = uracil, cytosine, adenine, hypoxanthine

RESISTANT AGAINST :

bacterial PMase
snake venom 5'-nucleotidase
bull semen 5'-nucleotidase

Scheme 6

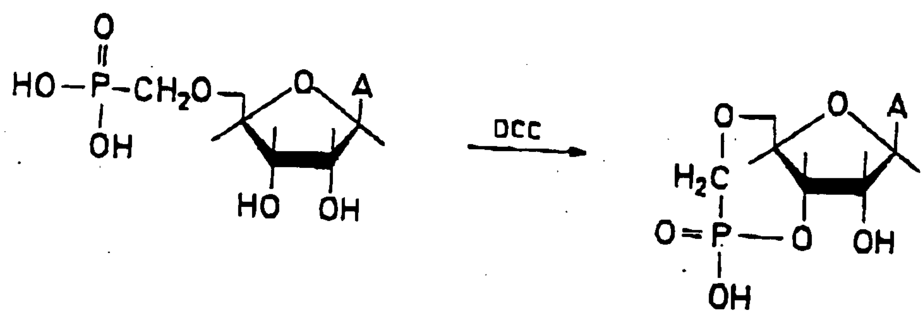
The 5'-ribonucleotide analogues thus prepared have many features in common with their natural counterparts: they possess two dissociable groups important for enzyme recognition and do not differ sterically too much from nucleotides. They are reasonably stable in acidic and alkaline solutions but decompose in boiling acetic acid. The expected stability towards dephosphorylating enzymes (bacterial alkaline phosphatase, intestinal alkaline phosphatase, seminal and snake venom 5'-nucleotidases) was fully confirmed. On the other hand, they behave like very potent competitive inhibitors of the two latter enzymes. The data compiled in Table 2 demonstrate the inhibitory action of three such analogues on seminal and snake venom 5'-nucleotidases (the both enzymes display also significant substrate inhibition; the corresponding data computed from Dixon-plots reflect relative inhibitory effect referred to the concentration of substrate and inhibitor).

In practical terms, an equimolar amount of the analogue fully inhibits the dephosphorylation of UMP under the assay conditions. The experiments on the binding of such nucleotide analogues on affinity support materials are now in progress.

Table 2. Inhibition of UMP dephosphorylation by nucleotide analogues.

5'-O-Phosphorylmethyl derivative of	$(\frac{i}{s})_{v/2}$	
	$s=5,65\times10^{-3}$M	$s=16,95\times10^{-3}$M
Snake venom 5'-nucleotidase		
Uridine	0,723	0,525
Cytidine	2,685	2,491
Adenosine	0,338	0,496
Bull semen 5'-nucleotidase		
Uridine	0,584	0,394
Cytidine	0,763	0,541
Adenosine	0,087	0,089

$(\frac{i}{s})_{v/2}$ ... inhibitor to substrate ratio for $v=v_o/2$, v_o ... initial rate of splitting in the absence of inhibitor.



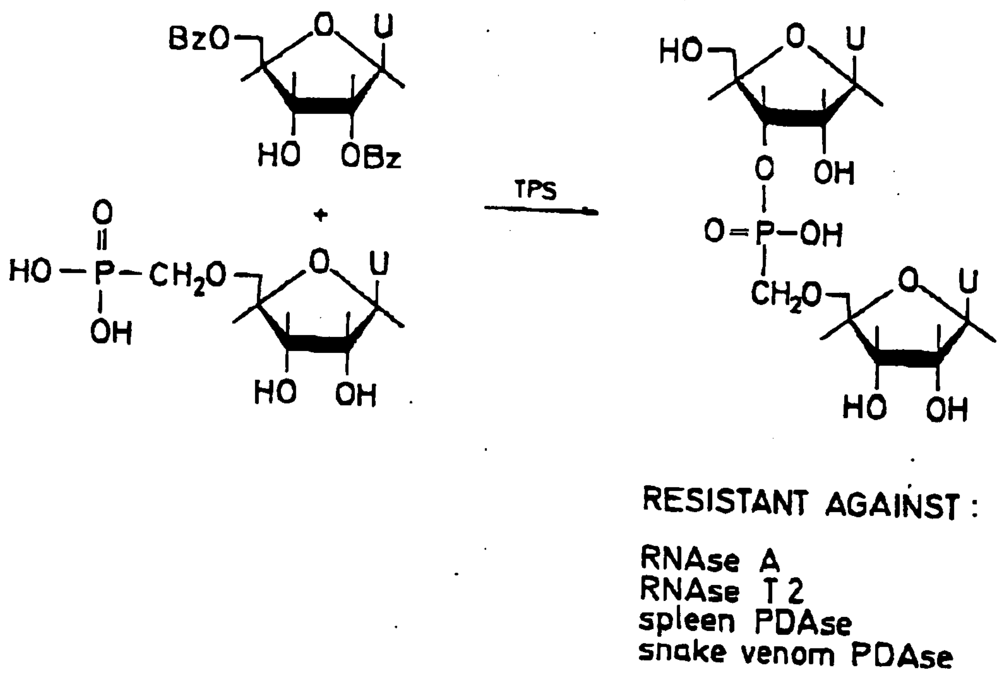
RESISTANT AGAINST:

dog heart 3',5'-cPDAse
spleen cPDAse
pollen cPDAse
snake venom PDAse
T 2 RNAse

Scheme 7

The above nucleotide analogues, (5′-deoxyribonucleoside-5′-yl)oxymethanephosphonic acids, can be used for further syntheses: the reaction of the adenosine derivative with N,N′-dicyclohexylcarbodiimide in dilute pyridine solution results in cAMP analogue (Scheme 7) containing a seven-membered cyclic phosphonate ring. This compound is quite resistant towards the cAMP-phosphodiesterase from dog heart as well as towards other phosphodiesterases. Its further implications are now under investigation.

Finally, a synthesis of a UpU-analogue was performed via classical phosphodiester approach (Scheme 8): 2′,5′-di-O-benzoyluridine on condensation with uridine analogue in the presence of TPS, followed by methanolysis, affords the desired compound in a fair yield. Necessarily, the analogue is quite stable not only against ribonucleases (pancreatic RNAse, T2-RNAse), but also against 3′- (calf spleen) or 5′- (snake venom) phosphodiesterases.



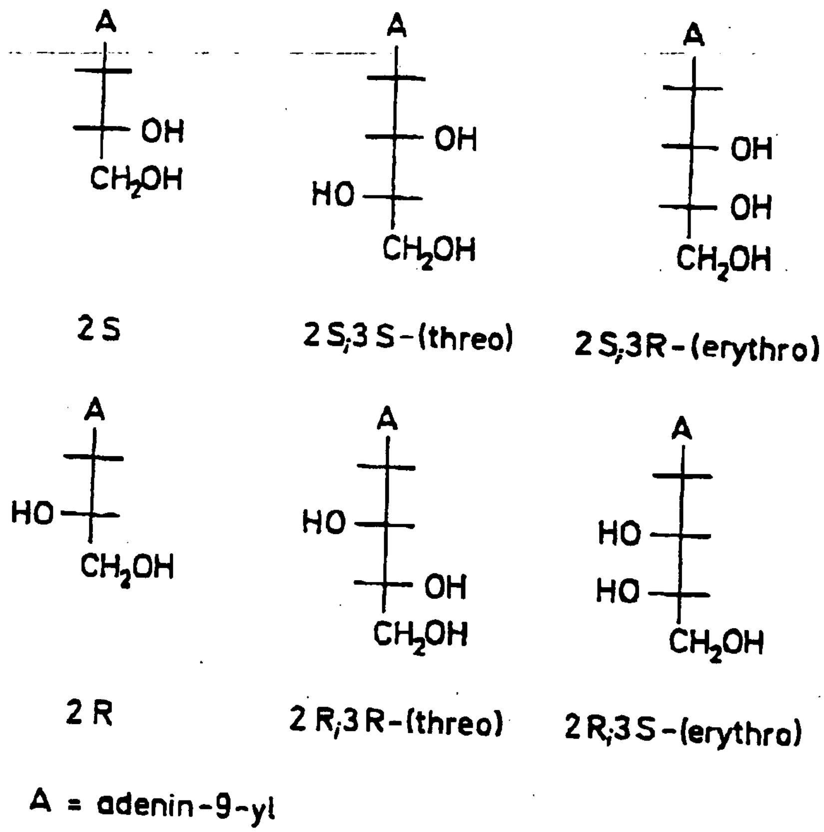
Scheme 8

The chemistry and biochemical implications of this type of nucleotide analogues is encouraging: the linkages are stable both chemically and enzymatically and the oligonucleotide analogues eventually obtained would almost surely exhibit different physico-chemical and biological properties dependent upon the steric changes involved. These and other problems including analogues of 3′-nucleotides, 2′-deoxyribonucleotides or 5′-diphosphates, are now under exhaustive investigation.

3. OPEN-CHAIN ANALOGUES OF NUCLEOTIDES

The chemistry of open-chain analogues of nucleic acids components (derivatives of nucleic acids bases bearing hydroxyl-containing aliphatic chain) has been investigated in this Laboratory in the recent years in connection with the hypothesis of minimum necessary similarity (36). The leading idea of this work is the assumption that such analogues might sufficiently differ from the natural metabolites to be stable under the in vivo conditions and, on the other hand, might fulfill the substrate requirements of other enzymes involved in the nucleic acids metabolism to display inhibitory or substrate activities. This assumption was confirmed by preliminary studies on some enzymes (37) and, mainly, by the recent finding of a substantial antiviral activity of two compounds: 9-(2-hydroxyethyloxymethyl)guanine ("acycloguanosine") (38) and 9-(S)-(2,3-dihydroxypropyl)adenine ("S-DHPA")(39,40). Also eritadenine (a natural hypocholesterolemic agent)(41) and 9-erythro-(2-hydroxy-3-nonyl)adenine ("EHNA", a powerful adenosine deaminase inhibitor)(42) belong to this category.

In our previous paper (37) we discovered the homochiral affinity of the cyclicphosphodiester derivatives of (S)-(2,3-dihydroxypropyl) uracil, -thymine or -adenine to some ribonucleases. This finding stimulated a detailed investigation of the homologous 2,3,4-trihydroxybutyl series of compounds with regard to their behaviour in enzymatic systems. Bearing two centres of chirality, the mentioned compounds can exist in two enantiomeric pairs (Scheme 9). The synthesis of parent "nucleoside analogues" has been already described elsewhere (43).

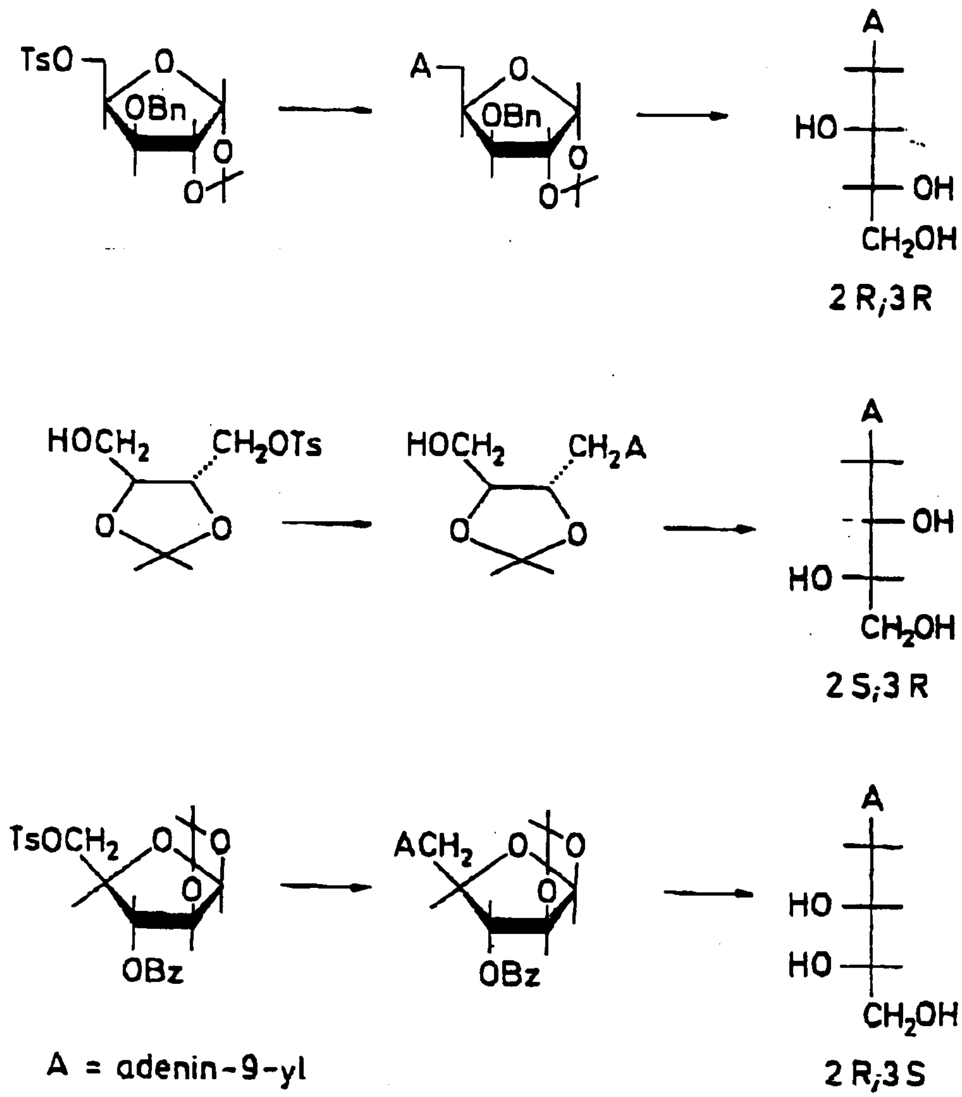


Scheme 9

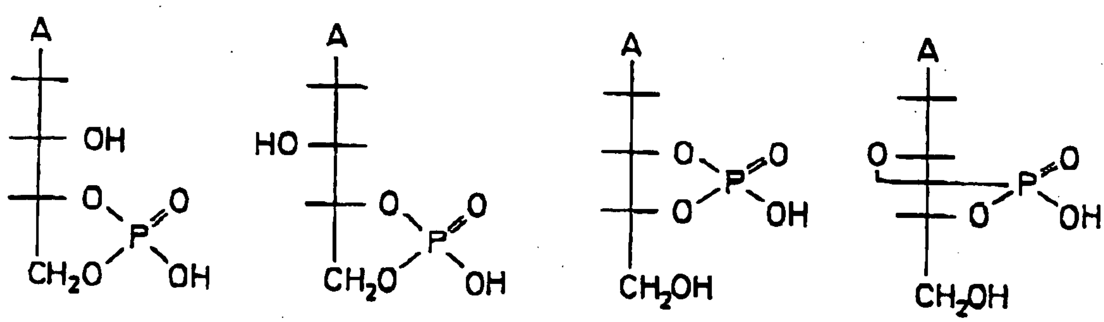
The assymetric syntheses (Scheme 10) start either from sugar derivatives by the degradative procedures, or, alternatively, from accesible optically active carboxylic acid derivatives by build-up pathways.

The phosphorylation of these compounds by phosphoryl chloride/triethyl phosphate procedure (44) affords mainly the 4′-phosphate accompanied by the 2′- and 3′-isomers. These isomers are easily separated by paper chromatography in borate-containing systems, or by HPLC technique. The preparative separation was achieved by anion-exchange chromatography on Dowex 1 resin. The phosphomonoesters thus obtained were easily converted to the cyclic phosphodiesters by the action of water-soluble carbodiimides. In all cases, only the five-membered phosphodiesters were detected; the separation of 2′,3′- and 3′,4′-isomers was finally performed by preparative paper chromatography and the constitution proved by the weak acidic cleavage to the known isomeric phosphomonoesters mentioned (Scheme 11).

These procedures were performed in two series of compounds, derived from uracil and adenine. The preliminary experiments revealed that the response to the ribonuclease T2 is markedly higher in the latter series and the further examinations were henceforth carried out with the adenine derivatives only. In this instance, the substrate activity of the 2′,3′- and 3′,4′-cyclic phosphates belonging to the two (erythro and threo) enantiomeric series was examined with non-specific ribonuclease T2 (Asp.oryzae). (The enzyme used was isolated according to the previous paper (45) and finally purified by isoelectric focusing; the preparation contained only traces of T1 RNAse and phosphomonoesterase activity.) As marker compounds,



Scheme 10



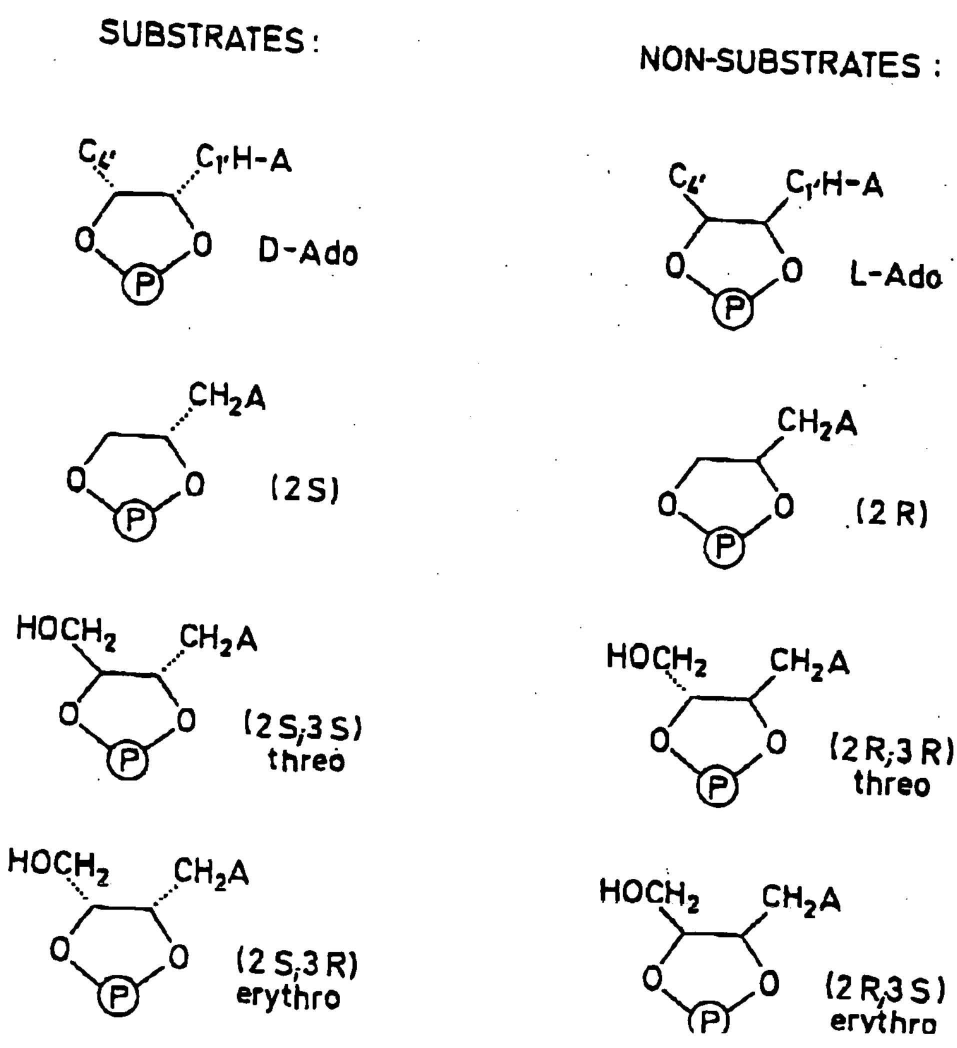
3,4-cyclic phosphates

2,3-cyclic phosphates

Scheme 11

the 2',3'-cyclic phosphates of adenosine, L-adenosine (46) and of the two enantiomers of 9-(2,3-dihydroxypropyl)adenine (36) were also included. It has been established that neither of the four 3',4'-cyclic phosphates underwent any change in the presence of the above enzyme. On the other hand, the results with 2',3'-cyclic phosphodiester derivatives (Scheme 12) indicate that in the enantiomeric series with the same absolute configuration at C(2')-atom as that of adenosine, both the 2,3-dihydroxypropyl- and the two 2,3,4-trihydroxybutyl derivatives do undergo ring-opening of the phosphodiester. The product which was identified in the latter series is exclusively the 3'-isomeric monophosphate. In contrast to these results, neither compound of the opposite absolute configuration is split by the ribonuclease T2. The homochirality of the reaction thus confirmed suggests that it is the whole molecule of the open-chain analogue, i.e. its base moiety, aliphatic chain and the phosphodiester cycle, which is involved in the ES-complex formation. Assuming approximately the same conformation of the latter residue, the comparison of the molecular models allows to suggest that the (S)-(2,3-dihydroxypropyl)derivative and both the (S,R)- and (S,S)-(2,3,4-trihydroxybutyl)derivatives can easily assume a conformation very similar to that of adenosine derivative as regards the mutual orientation of the adenine base and the phosphorus containing cyclic moiety. However, the enantiomers of the above mentioned three nucleotide analogues cannot approximate such a conformation (the nearest possible situation resembles the conformation of "1'-homoadenosine", i.e. an allitol derivative, which is also known to be resistant towards RNAse T2 (47)).

RNAse T2



The kinetic measurements performed so far with the four substrates are summarized in Table 3. The K_m constants calculated from the linear Lineweaver-Burke plots are very similar; in other words, as far as the K_m value can be regarded as a measure of the substrate affinity, the compounds tested exhibit affinity similar to the "natural" adenosine derivative. However, the maximum rate constants V_{max} differ dramatically within four orders of magnitude from this substrate. Whereas the three-carbon-chain derivative still exhibits a medium rate of splitting, the homologous 2,3,4-trihydroxybutyl derivative of the threo (2S,3S)-configuration is split very slowly; the erythro (2S,3R)-derivative is cleaved still more slowly and its kinetic parameters so far obtained are very inaccurate.

Table 3. Kinetic parameters of RNAse T2 splitting

2',3'-Cyclic phosphate	$K_m.10^3$ "M"	$V_{max}.10^6$ (mol.min^{-1})
Adenosine	3.561	63720.0
9-(S)-(2,3-Dihydroxypropyl) adenine	3.105	67.0
9-(2S,3S)-(2,3,4-Trihydroxybutyl)adenine	4.873	5.8
9-(2S,3R)-(2,3,4-Trihydroxybutyl)adenine	+	+

Anyway, it is perhaps possible to conclude that the presence and relative orientation of the substituent (CH_2OH) group at the 3'-carbon atom of the aliphatic chain brings an additional negative factor affecting the reaction at the active site of the enzyme. This seems to be understandable in the light of the results obtained with 9-(α-L-lyxofuranosyl)adenine derivative in which other hydroxylic function is introduced into the vicinity of the phosphorus atom and which is hydrolyzed more slowly than the adenosine derivative (48). Thus, the structure of the nucleoside derivative is optimal, since the energy required for its conformational change in the ES-complex is smaller than that required for the open-chain analogues with the freedom of rotation around the sp3-hybridized C(1')-atom. However, as proven by the above data, even the latter analogues are acceptable as substrates for the RNAse T2 and form the same type of product by the enzymatically catalyzed reaction.

The situation might be similar also with other enzymes and the open-chain analogues; it is however by no means of general validity: e.g., the above 2'-, 3'- or even 4'-monophosphates are not substrates for 5'-nucleotidases either from snake venom or from bull semen. Owing to the general interest in the open-chain analogues, other enzymes connected with nucleic acids metabolism should be closely investigated. On the other hand, the above examples demonstrate that such analogues are suitable for studies leading to better understanding the processes connected with the substrate requirements and mode of action of enzymes in general.

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Antisense Has Growing Pains

Efforts to develop antisense compounds as therapies for cancer, AIDS, and other diseases have encountered some unexpected questions about how the drugs really work

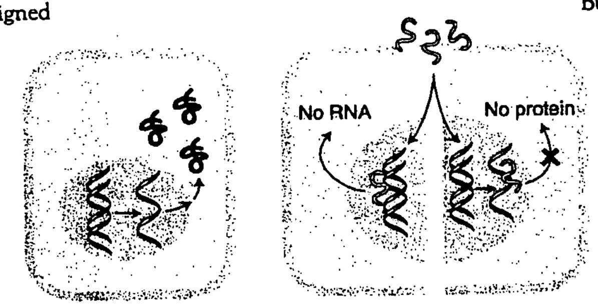
When *Science* named the gene-blocking technique known as antisense technology runner-up for its 1992 "Molecule of the Year," the accolade seemed well deserved. At the time, the technology appeared to offer a promising way to turn specific genes on or off at will. And that had made it potentially a powerful tool for uses ranging from fundamental molecular biology to the development of pharmaceuticals. Indeed, firms, both new and established, were rushing to exploit the technology to produce novel, rationally designed drugs for treating conditions ranging from genetic diseases to viral infections, including AIDS, and even cancer.

But during the past few years, the technique has run into unforeseen problems, and some of that early gloss has begun to wear off. Although several clinical trials have already begun, and there have been some promising results, researchers have encountered difficulties in getting antisense drugs—usually short pieces of DNA (called oligonucleotides) that have been designed to recognize and bind to specific genes—into target tissues. And potentially toxic side effects, including decreased blood clotting and cardiovascular problems such as increased blood pressure and decreased heart rate, have shown up in animal studies that have served as the basis for early human trials. But the biggest concern is that antisense compounds simply don't work the way researchers once thought they did.

"The assumption is that we are designing oligonucleotides that don't interact with anything besides [their targets]," says Cy Stein, an assistant professor of medicine and pharmacology at Columbia University's College of Physicians and Surgeons in New York City. "Many people are worried that a lot of the positive effects reported are not just antisense but other nonantisense mechanisms as well."

This uncertainty about what antisense drugs are doing inside the body has caused some experts in the field to argue that clinical trials have begun far too soon. "It is too early to take these things to human beings ... when we don't even know how they are working in a test tube," contends Ramaswamy Narayanan, who studies antisense drugs at Hoffmann-La Roche Inc. in New Jersey, but is not involved in any of the trials.

Others argue that even if the basic researchers haven't yet worked out the drugs' mechanisms of action, clinical trials are justified as long as the compounds show signs of efficacy. "As a clinician, what matters to me is if the drug works," says Jeffrey Holt, a pathologist at Vanderbilt University in Nashville, Tennessee, who is currently trying to use antisense DNA to fight advanced-stage breast cancer. "In medicine, people give drugs that we don't know the mechanism for." As one example, he cites aspirin, whose mode of action was not understood until relatively recently, even though it's been widely used for a century.



Holding on. In an untreated cell *(left)*, a gene's double-stranded DNA is transcribed into RNA, which then makes the protein *(green)*. Antisense drugs *(yellow)* are supposed to block this, by binding to the gene *(near right)* or the RNA *(far right)*. But do they?

Early promise

One reason antisense technology looked like the answer to drug designers' prayers is that it seemed to be simple and straightforward. During the first step of protein synthesis, in which genes are copied into RNA, only one strand of the double-helical DNA is so transcribed. The original idea, developed in the late 1970s and first published by Harvard Medical School researcher Paul Zamecnik, was to create a second RNA or DNA with a particular gene's complementary sequence—the so-called antisense molecule—that could recognize and bind to the RNA. This was supposed to prevent the RNA from manufacturing its protein, either directly or by causing it to be broken down by RNA-cutting enzymes. In the years since then, the technology has undergone several modifications, however.

To try and produce new drugs, researchers chemically string together a sequence of about 20 DNA bases—the oligonucleotide—that mirrors a short stretch of the gene scientists want to block. These may act either by binding to the RNAs, as the longer molecules do, or by binding directly to a target gene, thereby preventing it from being transcribed into RNA in the first place. (This latter approach is sometimes called "triplex" technology because a third DNA strand is being added to the two already in the DNA double helix.)

But however they work, such short oligonucleotides are much easier to synthesize than long antisense RNAs or DNAs. Researchers also made them more resistant to the many enzymes that break down nucleic acids by replacing a critical oxygen atom in each nucleotide building block with a sulfur atom. That's an important plus for a drug that has to be administered to a live human being, as it helps ensure that the drug will last long enough to do its job.

These modifications seemed to put drug designers on the right track: In initial tests with cultured cells, the sulfur-modified oligonucleotides, called phosphorothioates, appeared to work. For example, a team at Hybridon Inc., a biotech firm in Worcester, Massachusetts, found that one of their phosphorothioates, which they called GEM91, blocks replication of the AIDS virus, HIV-1, by targeting a viral life cycle gene called *gag*. "The antisense compound can suppress viral activity in vitro by up to 100%, depending on the concentration we use," says Sudhir Agrawal, vice president of drug discovery and chief scientific officer of the company. Other researchers also had early success in blocking reproduction of HIV-1 and other viruses with the sulfur-modified antisense constructs.

The successes quickly spurred the start-up of several biotech enterprises, such as Gilead Sciences Inc., an 8-year-old biotechnology company based in Foster City, California. "When we began, we said, 'Obviously from the literature, the technology works,' " recalls Richard Wagner, a molecular biologist at the company. "We thought that all we needed to do was bring in a few chemists and we were going to be rich."

But shortly after setting up shop, Gilead researchers realized it wouldn't be that simple. They quickly found that antisense

SOME CURRENT U.S. ANTISENSE CLINICAL TRIALS			
Company	**Disease**	**Rationale**	**Number of Patients**
Isis Pharmaceuticals	CMV retinitis in AIDS patients	Block CMV reproduction	200+
Isis Pharmaceuticals	Genital warts	Block human papilloma-virus reproduction	70+
Isis Pharmaceuticals	Kidney transplant rejection	Block immune cell activities	20 to 40
Isis Pharmaceuticals	Rheumatoid arthritis and other autoimmune diseases	Block immune cell activities	20 to 40 per disease
Lynx Therapeutics	Chronic myelogenous leukemia	Block cancer gene activities	50+
Hybridon	AIDS	Block HIV reproduction	125

compounds applied to a strain of human blood cells did not even get into the nucleus, the site of their RNA or DNA targets, Wagner explains. To get around that problem, they were forced to inject the compounds directly into the cells, a technique that works well in laboratories but cannot be applied to patients.

They did get some encouraging results, though: When they performed the injections, Gilead workers found that compounds directed at the *rev* or *gag* genes located in HIV-1 inhibited viral replication in the cells. In other experiments, antisense oligonucleotides targeted to the *c-myc* gene of blood cells from leukemic patients shut down cancer cell proliferation. But in both sets of experiments, yet another glitch cropped up.

To their surprise, researchers found that oligonucleotides they were using as controls, which couldn't recognize the *rev*, *gag*, or *c-myc* genes, either shut down virus replication or blocked cell proliferation almost as effectively as the ones they were testing as drugs. "While we could repeat many of the biological effects caused in cell culture, in every case our controls would show the same response," Wagner notes. "When we went back to the original papers, we found that often these controls were missing."

At first, Gilead researchers kept their concerns quiet. "There were a significant number of people claiming that these things worked," Wagner explains. "We really didn't want to go public with our negative results until we were sure that we weren't doing something wrong in our system." By the early 1990s, however, other researchers were echoing Wagner's concerns.

One example comes from Arthur Krieg of the University of Iowa, Iowa City, and his colleagues, who were attempting to develop antisense compounds that could be used to treat autoimmune diseases, such as rheumatoid arthritis, in which the immune system begins attacking the body's own tissues. "The B cells in autoimmune disease are hyperactive," Krieg explains. "We were trying to identify the genes responsible and shut them down."

When the researchers tried to inhibit B cells in culture with antisense DNA, however, the molecules turned B cell function up instead of down. That result was a mixed blessing, because it suggested that while the compounds tested would not be useful for treating autoimmune diseases, they might help buttress immune cell function in AIDS patients. But the Iowa team encountered an anomaly in their system similar to the one the Gilead workers had previously found. "Later, we got concerned as a number of controls also turned out to be B cell activators as well," Krieg recalls.

The immunologist, who says he worked "full time" to figure out what was causing this, came up with a solution earlier this year. In a paper published in the 6 April issue of *Nature*, Krieg and his colleagues reported evidence suggesting that antisense oligonucleotides mimic bacterial DNA in triggering a potent response by mammalian immune cells. They based this conclusion on experiments in which they showed that DNA fragments containing the two-base sequence CpG (where C stands for the nucleotide base cytosine, the G for guanine, and p for phosphate) activate mammalian B cells and natural killer cells in culture.

This only takes place, however, when the CpG motif lacks methyl groups. Because such sequences are common in bacterial DNA, but not in mammalian DNA, where most nucleotides have an attached methyl group, the immune response may be a way of defending against bacterial infections, Krieg suggests. The finding applies to antisense technology because antisense manufacturers don't usually add methyl groups to their synthetic oligonucleotides. Thus, mammalian immune systems that encounter such compounds with the CpG motif may be tricked into thinking they have been invaded by bacterial aliens and consequently spring into action.

Krieg suggests that this response could be useful clinically, but he says researchers need to be aware that the drugs are working directly on the immune system, rather than, say, targeting the AIDS virus itself. "I am firmly convinced that synthetic oligonucleotides, like the ones in clinical trials now, will make useful drugs for human beings," Krieg says. "But I don't think they are working through true antisense mechanisms."

Side effects in animals

Besides not always working by "true antisense mechanisms," the synthetic oligonucleotides have also caused side effects in experimental animals. When administered by one-time injection in high doses to monkeys, for example, several phosphorothioate drugs were lethal to some of the animals, for reasons that are not yet understood. In others, the oligonucleotides caused a transient decrease in the total number of two kinds of white blood cells as well as changes in blood pressure and heart rate, according to Hybridon's Agrawal. In addition, phosphorothioates have been found by Hybridon and Isis researchers to accumulate in the liver, kidneys, and bone marrow of animals, although the long-term effects of this deposition are not clear.

Some of these effects may be explained by the drugs' propensity to bind to proteins, says Columbia's Stein. At a recent meeting on the "Art of Antisense,"* molecular pharmacologist Stein presented some of his teams' findings on why the compounds often don't make it to the nucleus. They've found that they end up instead in the endosomes, small membrane-bound vesicles in the cytoplasm. This apparently occurs because the oligonucleotides tend to bind to proteins, which are themselves incorporated in the endosomes. "Many cell types protect themselves by sequestering oligos in intracellular compartments," Stein says, but this could also contribute to the deposition of the drugs in liver and kidney.

In addition to getting entangled by proteins inside cells, the Columbia researcher found that many synthetic oligonucleotides, because of their highly negative charge, get hung up on proteins outside cells as well. Among these are growth factors and cell anchoring proteins such as fibronectin and laminin. The result is that antisense compounds block cell migration and adhesion to underlying tissue in vitro—an effect that may interfere with wound healing and arterial wall repair in living animals, Stein says.

Hybridon's Agrawal maintains, however, that the cardiovascular and other effects seen in animals can be minimized in patients by using low doses of the compounds and administering them gradually by continuous intravenous injection. That seems to be borne out by the early results of Hybridon's

* The meeting, which was sponsored by *Nature Medicine*, was held in New Orleans on 21 and 22 September.

clinical trial of GEM91 in AIDS patients, he told participants in the antisense meeting. Agrawal also reported that patients getting the higher doses are showing signs of clinical improvement in that their viral counts drop a few days after the treatments, although it is far too soon to tell whether this translates into improved survival. To Agrawal, it doesn't matter how the drugs work, if they end up helping AIDS patients. "Despite all the other properties [in addition to actual gene targeting], we feel that if we find an antisense effect ... then we have a new drug," Agrawal says.

Looking to the future

Agrawal is not the only one who hasn't lost faith in the technology. Biotechnology representatives argue that the problems turning up with antisense oligonucleotides are common in drug development, especially when untested, new technologies are being explored. "Every new technology starts at the bottom, in essence, getting your foot in the door," says Gerald Zon, vice president of medicinal chemistry at Lynx Therapeutics Inc., a biotech company in Hayward, California. He notes that every new drug has negative effects that must be weighed against clinical benefits. The answer, he says, is to design better second- and third-generation drugs in order to boost drug efficacy while, at the same time, minimizing unwanted side effects.

Indeed, researchers at companies such as Hybridon, Isis, and Gilead say they are applying the lessons they are learning from the animal studies and early clinical trials to try to come up with better and less toxic compounds. The options they are exploring include modifying the structures of oligonucleotides so that they bind less readily to proteins or more readily to their target genes. All three companies are also generating fat-soluble delivery molecules called cationic liposomes. The researchers hope these lipid-loving shuttles will help antisense compounds break through cellular barriers that prevent entrance into the nucleus.

These new compounds and delivery systems carry no guarantees that they will be any better than the phosphorothioates used in the current clinical trials. But even some of the critics, such as Stein, agree the field still holds great promise, as long as the researchers recognize that antisense drugs don't always work the way they are supposed to. "My guess is that we will find that the current generation of phosphorothioates are extremely active biological molecules," Stein concludes, "and that they work by many mechanisms, of which antisense is one. The truth is that we'll have to wait and see. None of us really knows what is going to come out of it."

–Trisha Gura

Trisha Gura is a reporter on leave from the Chicago Tribune.

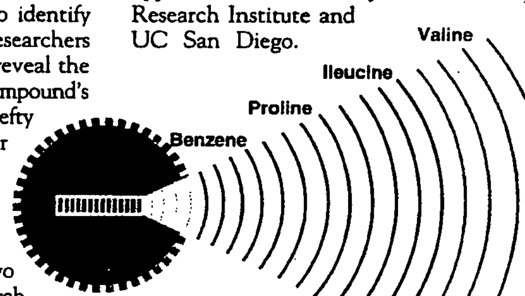
CHEMISTRY

Radio Tags Speed Compound Synthesis

Like aging computers, it doesn't take long for scientific techniques to seem slow and cumbersome. Take combinatorial chemistry. When it was introduced a few years ago, it was the supercomputer of chemical synthesis. The technique allows chemists to quickly paste together several different chemical building blocks into millions of combinations, in hopes that one will prove to be a new drug or a useful material. To identify each one of the new compounds, researchers typically affix chemical tags that reveal the unique arrangement of each compound's components. But these tags carry a hefty price: Their use doubles the number of chemical steps—and the time—involved in the assembly process, and their fragility prevents the synthesis of some compounds.

In the past 2 weeks, however, two separate groups of California researchers have unveiled a faster and more agile model. By replacing chemical ID tags with tiny radio-emitting microchips, they appear to have overcome both of the problems inherent in the old one. "The upshot is that it makes the whole process of drug discovery more efficient," says Rob Armstrong, a chemist at the University of California (UC), Los Angeles, who led one of the research groups, which includes scientists from Ontogen Corp. in Carlsbad, California. "This has the potential to be a significant advance in simplifying the encoding process," adds Michael Pavia, who heads combinatorial research at Sphinx Pharmaceuticals in Cambridge, Massachusetts. The technique not only saves time, says Pavia, "it gives you a wider range of chemical diversity to select from in building your new molecules."

Armstrong's group presented its findings at last week's meeting of the Western Biotech Conference in San Diego, as did the second team, led by Michael Nova at IRORI Quantum Microchemistry in La Jolla, California, and K. C. Nicolaou, who holds dual appointments at the La Jolla–based Scripps Research Institute and UC San Diego.



Inventory tracking. A radio-emitting microchip can encode the identity of molecules grown on surrounding beads.

The IRORI group was, however, the first in print, with a paper in the 15 October issue of *Angewandte Chemie*.

Both techniques add considerable power to combinatorial chemistry, which already made traditional synthetic chemistry look like an old IBM punch card. Traditionally, novel compounds are synthesized one at a time, but combinatorial chemists create huge numbers in a single process by assembling a few chemical building blocks—each of which has a corresponding ID tag, such as a short nucleotide sequence—in all possible combinations. Chemists need these tags to decipher the makeup of compounds that show promise in an assay, such as the ability to kill cancer cells (*Science*, 3 June 1994, p. 1399).

But because a tag has to be added with each building block, assembling a 10-component molecule actually involves at least 20 time-consuming chemical steps. And the technique runs into trouble when creating small organic molecules, which constitute most of today's drugs. Some of the synthetic reactions involve potent reagents, such as hydrofluoric acid, which can rip ID tags apart.

The new microchip tags appear to solve both these problems at once. A chip, which emits a binary code, is inserted into a mesh capsule loaded with polymer beads—the "seeds" to which combinatorial building blocks are added by dunking the capsule in a series of beakers. In the Ontogen approach, a nearby radio scanner registers both the identity of a capsule and the contents of each beaker it enters. These data are uploaded to a computer that keeps track of the order of building blocks in the growing molecule. In the IRORI approach, the information is stored on the microchip itself, using a transmitter that writes the information to the chip. The information is uploaded to the computer only when the assembly run is complete.

By eliminating the chemical tags, both approaches do away with half the synthetic steps involved, yet end up with an instantly available computer record of the precise structure of the compounds in each capsule. Moreover, says Nicolaou, "now you are free to use any chemistry you want to build your molecules." Speed and flexibility—for chemists, it's a winning combination.

–Robert F. Service

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Antisense oligonucleotide therapeutics: drug delivery and targeting

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Abstract

Oligonucleotide(ON)-based therapy, although in an early stage of development, promises to provide new and highly specific tools for the treatment of human diseases such as virus-associated illnesses and cancers. Like gene therapy, ON therapy is a rapidly growing field with great therapeutic potential. However, the two types of therapy differ fundamentally in their approach. In gene therapy, missing or defective genes are added or replaced with functional versions, while in ON therapy, existing but abnormally expressed genes are inhibited. In this article, we will focus on ON therapy with an emphasis on issues related to ON drug delivery, stability, and targeting. ONs have several advantages over traditional drugs, notably their exquisite specificity to target sites and their ease of design. However, their effective use has been limited due to several problems. For example, naturally occuring ONs contain phosphodiester backbones that are easily degraded in a biological environment and therefore must be protected or modified to render stability. In addition, because of their large molecular size and charge, these compounds are poorly taken up by cells and therefore may not reach their target site. Moreover, problems associated with cellular targeting, potential toxicity, and affinity of ONs to the target sites pose major challenges to the successful utilization of these compounds. Here we shall examine recent findings, relative advantages and disadvantages of various ON delivery methods, as well as the common pitfalls peculiar to each strategy.

Keywords: Antisense; Oligonucleotide; Gene; Delivery; Stability; Targeting; Uptake; Endocytosis

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Abbreviations: DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride; EGF, epidermal growth factor; ICAM-1, intercellular adhesion molecule 1; ON, oligonucleotide; PLL, poly(L-lysine); VSV, vesicular stomatitis virus; HIV, human immunodeficiency virus; 5′-UTR, 5′-untranslated region.

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1. Introduction

Antisense refers to the use of short, single-stranded, synthetic ONs to inhibit gene expression [1,2]. These compounds are designed to be complementary to the coding (sense) sequence of RNA inside the cell. After hybridization to target sequences, translational arrest occurs via one of several putative mechanisms. The first is ribosomal blockade where the antisense molecule hybridizes to the sense sequence and prevents the ribosome from reading the mRNA code, resulting in production of a defective nonfunctional protein. The second is the specific cleavage of RNA strand by activated RNase H following RNA-ON hybridization. This cleavage results in destruction of the coding message and inhibition of protein synthesis. The third is the competition between the ribosome and the antisense ON for binding to the 5′-untranslated region (5′-UTR) of the mRNA. Binding of the ON to the 5′-UTR can also result in activation of RNase H and subsequent cleavage of the mRNA. Finally, synthesis of fully mature mRNA in the cytosol can also be prevented at the level of RNA transcription, splicing, processing, or transport across the nuclear membrane. For example, ON can bind to the complementary sequence on nuclear DNA, forming triplex DNA which selectively inhibits DNA transcription [3,4]. The well known Watson-Crick base pairing rules govern the interaction between the ON and the RNA while the Hoogsteen base pairing rules dictate the interaction between the ON and the DNA [1,5]. Such interactions allow the potential to target ON to virtually any gene of interest.

2. Potential advantages of antisense therapy

As stated above, the striking advantage of the antisense approach is its potential for specificity. Conventional chemotherapy for conditions such as neoplastic and virus-related diseases is fraught with systemic toxicity. The therapeutic window of chemotherapeutic agents is relatively narrow, which reflects the agents' inability to distinguish between normal and diseased cells. A drug is specific if it has a strong binding affinity for its target receptor relative to other binding sites. Antisense ONs have the potential to be many orders of magnitude more specific than traditional drugs due to their greater number of interactions with any particular target site [6]. Since specificity is proportional to the number of interactions between a drug and its receptor, specificity of an ON is expected to depend on its length. In theory, an ON of more than 15–17 nucleotides in length could have the base pairing specificity to interact with only one target gene within the entire human genome [7]. Thus, antisense ONs have a potential specificity that could serve as powerful research tool for the study of specific gene function and as therapeutic agents for disease-causing genes.

Another advantage of the antisense approach is the relative simplicity in which the drugs can be rationally designed. Traditionally, drug design strategies have often focused on identifying protein functions and their interactions with target receptors. The complex three-dimensional structures of both protein drugs and receptors has made drug design a long and difficult process. Not only do proteins possess multiple domains of varying functions, but divergent proteins may share common domains. Although the knowledge of protein structure proceeds apace, the understanding of structure-function relationships still lags behind. Therefore, identifying the interactions has been relatively empirical and the specificity of interactions has been limited. In contrast, the interaction between the drug and

the target mRNA in the antisense approach is governed primarily by the sequence of the target, although drug accessibility to the target sites may be limited by the complex structure of the mRNA. Nonetheless, antisense drugs designed to target various regions of mRNA have been shown to be effective in inducing hybridization arrest of translation. For examples, ONs targeting the start codon and extending upstream or downstream have been shown to be effective in inhibiting translation [8,9]. Likewise, ONs that are complementary to the splice sites have proved effective, but internally translated regions are less effective [8,9].

Pathologic disorders that are currently targeted by antisense therapeutics include viral infections, inflammatory disorders, cardiovascular diseases, cancers, genetic disorders, and autoimmune diseases. Examples of these applications are listed in Table 1. Most work to date on antisense pharmaceuticals has been focused on viral diseases for which only inadequate conventional therapy exists. The major viral targets include HIV [10,11], herpes simplex [19], influenza [20], and human cytomegalovirus [14]. Encouraging results have been obtained with cell culture and animal models of viral infection. Another interesting application for antisense therapy has been in the field of cancer. By now, many of the most important oncogenes have been cloned or sequenced. Those sequences allow one to design antisense agents that can down-regulate the expression of oncogenes known to be involved in the transformation of tumor. The potential antisense approach to cancer therapy that would be highly selective for transformed cells is based on the fact that some malignant cells have specific activating mutations in certain oncogenes such as *ras* or translocations as in *myc* and *bcl-1*. These mutations provide a

Table 1
Inhibition of gene expression by antisense oligonucleotides

System	Cell type	Reference
Viruses		
Human immunodeficiency virus	H-T cells	7
	H9 cells	8
	CZM cells	8
Human T-cell lymphotropic virus III	H9 cells	10
Human cytomegalovirus	NHDF cells	11
Vesicular stomatitis virus	L929 cells	12–14
Hepatitis B	Alexander	15
Hepes simplex virus	Vero cells	16
Influenza virus	MDCK cells	17
Rous sarcoma virus	Fibroblasts	18
Simian virus 40	MDCK cells	19
Mammalian genes		
Multiple drug resistance	MCF-1 cells	20
Proliferating cell nuclear antigen	3T3 cells	21
T cell receptor	T cells	22
Colony-stimulating factors	Endothelial cells	22
	FL-*ras*/*myc* cells	23
β-Globin	Reticulocytes	24
Interleukins	T-lymphocytes	25
	Monocytes	26
cAMP kinase	HL-60 cells	27
bcl-2	L697 cells	28
c-myb	Mononuclear cells	29
c-myc	T-lymphocytes	30
	HL-60 cells	31
	Burkitt cells	32
N-*ras*	T15 cells	33

therapeutic window that allow one to knock out cells containing those specific mutations.

Despite the apparent promise of the antisense approach, the design of therapeutically effective ONs involves a unique set of problems which must be overcome. These problems are discussed below.

3. Can antisense work in living systems?

There are numerous studies demonstrating the effectiveness of antisense ONs in various cell culture systems. However, several key questions remain, the most obvious one being "Can the antisense approach work in vivo?". This question has often been posed in different forms depending on the background of the person asking the question. From a drug delivery standpoint, the key question is often addressed like "How can antisense ONs be targeted to diseased cells, sparing normal cells?" or "How can antisense ONs be effectively delivered into the intracellular target sites where they can then exert their action?". The first question may be readily answered with the following postuate. If the antisense ONs exhibit no cellular toxicity or non-specific antisense activity, then targeting at the cellular level would not be necessary; that is, all cells could be exposed to antisense ONs. In principle, the exquisite specificity of antisense ONs implies that these compounds are less likely to cause toxic side effects in comparison to conventional drugs. However, several recent studies appear to suggest that cellular toxicity and non-specific activity of antisense ONs can occur (albeit in cell culture systems) [12,36–38]. To demonstrate antisense activity, ONs that are not complementary to the target RNA are usually used as controls. An antisense activity is implicated if the antisense ON inhibits better than the controls. However, frequently the control ONs inhibit as well or better than the antisense ON [39,40]. Non-specific toxicity of antisense ONs has also been suggested as a result of their degradative products in different cell types, particularly the hematopoietic cells [37,38]. In addition, non-antisense action has been shown for ONs carrying a small sequence of four consecutive guanosines (a G-4 tract) [36]. Non-complementary ONs carrying this sequence effectively inhibited cell proliferation and viral replication in cell culture systems [36]. However, it should be pointed out that the in vitro effects of ONs may not necessarily reflect their in vivo effects, and there are studies which indicate the relative safety of antisense ONs in vivo [39,40]. On the other hand, non-specific side effects of ONs have also been reported in mice [41]. Antisense ONs targeted against rel A transcription factor were found to induce thrombocytopenia and renal failure. The mechanism underlying this toxicity is not known, however this result suggests that ONs can have non-specific actions and may cause toxic side effects in vivo.

The second question "How can antisense ONs be effectively delivered into cells?" is equally difficult to answer. Most antisense ONs are poorly taken up by cells due to their hydrophilic nature and large molecular structure. In many cases, biological antisense activity can only be achieved in the presense of transfer vectors such as cationic lipids and liposomes. The obviousness of this problem, however, does not necessarily diminish the potential use of ONs in vivo, and there are few examples of successful in vivo treatment in the absence of specialized delivery systems [44,45]. Much effort has been made with some success to chemically modify ON and to develop carrier vectors for effective delivery of antisense ONs. These studies are detailed below.

4. Barriers to potent antisense activity

Considering the various obstacles that the antisense ONs must encounter prior to their action, it is quite remarkable or perhaps surprising that the desired activity of ONs is observed. First, the ONs must find their way to target cells where they must then penetrate the plasma membrane to reach their target site in the cytoplasm or nucleus. Second, once inside the cell the ON must be able to withstand enzymatic degradation presented by various endogenous nucleases. Third, the ON must be able to find and then bind specifically to its intended target site in order to inhibit expression of the disease-causing

gene. In addition, the ON must avoid binding to non-target sites as this may produce undesirable side effects. The observed activity of ONs in cell culture was previously thought to imply that these potential barriers were insignificant. However, careful analysis of recent studies indicates that these barriers are indeed real and many of these barriers can become key determinants in the successful outcome of antisense therapy.

4.1. Stability and chemical modification

The initial successful demonstrations of the antisense strategy in cell culture employed the naturally occuring phosphodiester ONs [8,21]. However, it was soon realized that phosphodiester ONs are easily degraded in cell culture medium containing serum due to 3′-exonuclease digestion [46]. Consequently, the antisense effects could only be observed if high ON concentrations (up to 100 μM) were used [47]. Protection from degradation can be achieved by the use of a "3′-end cap" strategy in which nuclease-resistant linkages are substituted for phosphodiester linkages at the 3′ end of the ON [46]. Alternatively, ONs containing a 3′-terminal hairpin-like structure were found to exhibit improved resistance to exonuclease digestion [48].

Phosphodiester ONs are not only rapidly degraded in serum– the first biological medium likely to be encountered during antisense therapy–but once the ONs enter cells, they can be further degraded by cellular endonucleases. Neither 3′-end caps nor 5′-end caps protect ONs from degradation in HeLa cell extracts [49]. Several other studies have also shown that phosphodiester ONs are rapidly degraded after microinjection into cells [50,51]. Thus, phosphodiester ONs are poor candidates for use as therapeutic agents in vivo. Consequently, a number of chemical modifications have been made to improve enzymatic stability of these compounds while preserving their ability to hybridize to cognate targets. The most commonly used are the first-generation analogs that possess modifications of the phosphodiester backbone. Examples of these include the phosphorothioate and phosphorodithioate analogs which have sulfur substituted for one or both nonbridging oxygens,

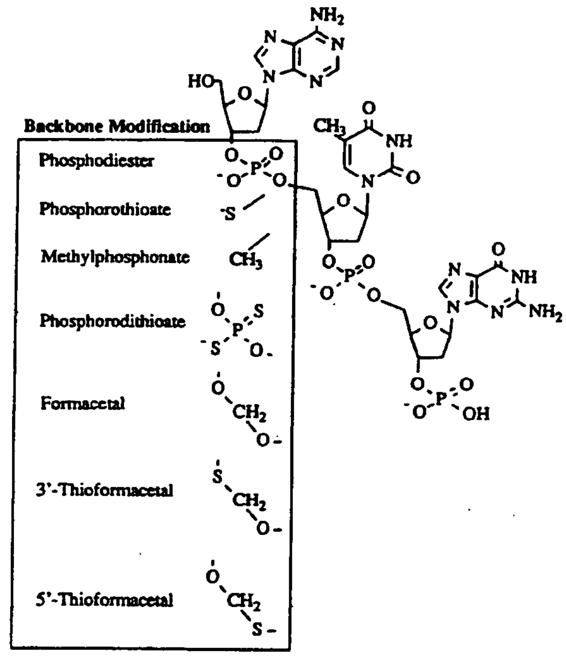

Fig. 1. Oligodeoxynucleotide backbone modifications.

and methylphosphonate analogs which have a methyl group substituted for one of the non-bridging oxygens (see Fig. 1). The synthesis of these modifications yields two isomers at each position, giving 2^n diastereomers, where n is the number of linkages [52]. In general, these modified ONs are less susceptible to nuclease digestion and, for the most part, exhibit greater antisense activity than their parent compounds (see [53] for review). Phosphorothioate ONs retain their negative charge groups in the phosphodiester backbone and have the ability to induce mRNA degradation via RNase H. However, these compounds, when compared to their unmodified counterparts, tend to have lower binding affinity to their target sequences due, possibly, to diastereomer formation [54]. Moreover, these compounds have been reported to exhibit non-sequence-specific activity [12,36]. Unlike phosphothioates, methylphosphonates are electrically neutral and have been suggested to have greater cellular uptake than unmodified ONs (see further discussion in section 4.2). However, these compounds are ineffective in

some cell lines. This may be due to the formation of diastereomers or the inability of methylphosphonates to induce mRNA degradation via RNase H. To avoid the problem of chirality and in attempts to improve the potency of antisense ONs, new backbone linkages are clearly desirable.

Other types of backbone modifications that have been employed to improve ON stability are formacetal and thioformacetal ONs. The formacetal linkage is created by replacing the central phosphorus with a methylene group (Fig. 1). This linkage is isosteric to a phosphodiester in that it contains free rotation about the bond of the linkage. The formacetal ONs possess base pairing specificity identical to that of the phosphodiester ONs but with slightly less affinity [55]. Further substitution of either of the bridging oxygens in the formacetal linkage with a sulfur yields a thioformacetal linkage (Fig. 1). ONs containing the 3′-thioformacetal linkages hybridize to RNA with greater affinity as compared to phosphodiester ONs, however, ONs with 5′-thioformacetal linkages hybridize to RNA poorly [55,56]. Therefore, the 3′-thioformacetal linkage is a strong candidate as a replacement for the phosphodiester linkage in ONs, conferring higher affinity and resistance to nuclease degradation.

4.2. Permeation and enhancement

When added directly to cells in culture, only 1–2% of the added ONs become cell-associated [57,58]. Consequently, high concentrations of ONs are generally required for activity. Therefore, enhanced ON uptake is a critical consideration in developing these agents for therapeutic applications.

Cellular uptake of ONs is an energy-dependent process and can be inhibited by treating the cells with metabolic inhibitors or by lowering the temperature [54,58–61].

This transport across the membrane takes place in a saturable and sequence-independent manner. Any sequence or size of ribo- and deoxyribonucleotide was demonstrated to compete with labeled ON for uptake [59]. The uptake is endocytic and appears to be mediated by membrane receptor proteins. Loke et al. [59] described a 80-kDa membrane protein as a receptor for ON, while Bennett et al. [62] reported a 30-kDa protein responsible for cellular uptake of DNA. Yakubov et al. [60] observed two labeled protein bands on gel electrophoresis following incubation of their cells with radiolabeled ON; one of these proteins corresponds to that described by Loke et al. [59]. Scatchard analysis of ON binding revealed single dissociation constants in the low nanomolar range and with a binding capacity of approximately 20000–100000 binding sites per cell [63]. However, others have argued that ON binding to membrane proteins is relatively nonspecific and is mostly charge associated, consistent with adsorptive endocytosis or fluid-phase pinocytosis. The latter should be true when the ON concentration exceeds the apparent K_d [60,64] of ON binding to the membrane binding protein. In most cases in which antisense effects are observed, ON concentrations are routinely in the range of 1–100 μM. Furthermore, inhibitors of receptor-mediated endocytosis have no significant effect on internalization of ONs in Rauscher cells [58]. Even more interestingly, an anion channel blocker, 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid, was found to inhibit ON uptake [58]. Whether channels are used in mammalian cells for the transport of nucleic acids have not been demonstrated.

Phosphorothioate ONs readily compete with unmodified ONs for uptake [54,59]. Uptake characteristics of phosphorothioate and unmodified ONs are similar and their internalized fluorescent probes exhibit a punctate fluorescence pattern indicating endocytosis. A comparative study of the uptake of 5′-acridine-linked 7-mer oligodeoxy-thymidylates by HL-60 cells showed that the phosphorothioate ON was taken up much more slowly than the corresponding normal ON [54]. In another study, however, the rate of uptake of both normal and phosphorothioate 28-mer anti-*rev* ON in lymphocytes was found to be comparable [65]. It is likely that the difference in probe size, cell type, and conditions used may contribute to this discrepency.

In contrast to phosphorothioates, methylphosphonates do not compete with unmodified ONs for uptake, suggesting a different transport

mechanism. Because methylphosphonates lack charged groups and are more hydrophobic as compared to normal ONs, and since phosphorothioates retain the same negative charge, it was suggested that these molecules may be passively transported across cell membranes [66]. However, studies by Akhtar et al. [61] and Shoji et al. [64] indicate that this is not the case. Passive diffusion of methylphosphonates across phospholipid membranes was found to be very slow (efflux $t_{1/2} > 4$ days) and was not significantly different from that of unmodified or phosphorothioate ONs [61]. In another study, Shoji et al. [64] demonstrated that cellular uptake of fluorescently labeled methylphosphonates was highly temperature dependent and exhibited intracellular punctate pattern, similar to that observed with unmodified or phosphorothioate ONs. Although this suggests an energy dependent endocytic process, excess unlabeled ONs (methylphosphonate or unmodified) did not compete for uptake.

Several approaches have been developed to improve cellular uptake of ONs. These include inclusion of ONs into liposomes or attaching them covalently or electrostatically to specific or nonspecific carriers. Some of these methods are summarized here and in Table 2.

4.2.1. Liposome-mediated antisense delivery

Liposomes have been most widely used to aid cellular uptake of ONs. Most liposomes used for this purpose are cationic liposomes which can form stable complexes with the polyanionic ONs. These liposomes consist mainly of a positively charged lipid, most notably *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride or DOTMA, and a co-lipid, e.g., dioleylphosphatidylethanolamine, to aid cytoplasmic delivery of the polynucleotides [90]. Recently, several different types of cationic lipids have been developed including lipofectin, quaternary ammonium compounds, cationic derivatives of cholesterol-diacyl glycerol and lipid derivative of polyamines (see reviews in [91,92]). Liposomes can be designed in various ways to provide protection from nuclease degradation, to target specific cell types, and to increase cellular uptake. In this review we will focus on the use of liposomes for the delivery and targeting of antisense ONs (see [93] for further discssion of the subject). Phosphorothioate antisense ONs complexed to cationic liposomes inhibited the expression of the ICAM-1 adhesion molecules in human lung carcinoma cells [67]. Several other research groups similarly demonstrated that, in the presence of cationic liposomes, ONs had strong antisense activity, while in the absence of cationic liposomes, no activity was observed [68–71]. These observations strengthen the argument that ONs do not normally cross cell membranes to any meaningful extent.

Targeting liposomes to specific cell types can be achieved by attaching an antibody or some

Table 2
Enhancement of antisense oligonucleotide uptake

Carrier system	Mechanism	Reference
Liposomes		
Cationic	Adsorptive endocytosis	67–71
pH-sensitive	Non-specific endocytosis/ Endosomal membrane fusion	72, 73
Immunoliposomes	Receptor-mediated endocytosis	74–76
Sendai virus-derived liposomes	Plasma membrane fusion	77, 78
Poly(L-lysine)	Adsorptive endocytosis	16, 79–82
Cholesterol	Unknown	83, 84
Phospholipids	Unknown	17
Avidin	Adsorptive endocytosis	85
Acridine	Intercalation	86
Streptolysin O	Pore formation	87
Divalent cations	Charge neutralization (?)	88, 89

other targeting ligand onto the liposomal membrane. Liposomes containing unmodified antisense ONs complementary to the translation initiation region of VSV N protein mRNA enhanced a sequence-specific and dose-dependent reduction in virus titer when targeted to L929 cells with H2K-specific antibodies [74]. *c-myc* mRNA specific antisense ONs delivered in the same way induced an antiproliferative activity [75]. The incubation of the targeted cells with the appropriate antibodies was required in these cases. Similarly, liposomes targeted with anti-CD3 antibodies, containing an antisense RNA to the HIV env region, inhibited viral protein expression [76].

Most liposomes are internalized by endocytosis where they eventually fuse with lysosomes. pH-sensitive or fusogenic liposomes have been developed as a means to circumvent this problem [72,94]. Prior to fusing with lysosomes, the pH of the endocytic vesicles is reduced and the liposomes destabilize the membranes. Using this approach, Ropert et al. [73] showed that antisense ONs against Friend leukemia virus encapsulated in pH-sensitive lipsomes inhibited the formation of viral foci more effectively than when associated with non-pH-sensitive liposomes. Although cytoplasmic delivery from pH-sensitive liposomes is more efficient, such delivery still accounts for only 0.01–10% of the liposome contents that become cell associated [72].

4.2.2. Polylysine-mediated antisense delivery

Poly(L-lysine (PLL), a well-known polycationic drug carrier [95], has been used to facilitate cellular uptake of various drugs including antisense ONs [16,79,80]. Using VSV-infected L929 cells as a model system, Lemaitre et al. [16] and Leonetti et al. [79] demonstrated that ONs complementary to viral nucleocapsid initiation site or to viral genomic RNA sequences promoted a sequence-specific and dose-dependent antiviral activity when administered as PLL conjugates. Antiviral activities of such conjugates were observed at concentrations below 1 μM while non-conjugated ONs were equally active when used at concentrations greater than 50 μM. Likewise, PLL-conjugated ONs complementary to a HIV-1 splice site inhibited cytopathic effects at much lower concentrations than non-conjugated phosphodiester or methylphosphonate ONs [80]. A similar approach was successfully used to develop an antiproliferative activity with anti-*c-myc* ONs [81] or to decrease the cytopathic effects of HIV-1 in de novo-infected MT4 T-lymphocytes [82]. In the *c-myc* study, nonconjugated ONs were only active when serum nucleases were absent while the PLL conjugates were active even in the presence of nucleases. This increased stability is believed to be a result of steric hindrance provided by the PLL moiety and by 3′ ON modification.

The uptake mechanisms of PLL-ON conjugates were investigated using flow cytometric analysis and fluorescently tagged ONs [96]. Both the extent and rate of uptake were increased when ONs were coupled to PLL. The uptake process exhibited endocytosis characteristics in that intracellular fluorescence was punctate, uptake was temperature- and energy-dependent, and lysosomatropic agents prevented the sequence-specific cytotoxicity of the ON. PLL has been shown to be internalized by cells through non-specific adsorptive endocytosis [95]. The polycationic PLL interacts nonspecifically with negatively charged molecules on the cell membrane and is internalized along with those molecules. Such high-capacity interactions could explain the efficacy of PLL-ON conjugates. The intracellular fate of the PLL conjugates remains to be delineated; proteolysis of PLL probably takes place in endocytic vesicles since poly(D-lysine) non-degradable conjugates do not show any biological activity.

The usefulness of PLL as a drug carrier may be limited by its potential cytotoxicity and non-specificity. Large molecular weight PLL is cytotoxic even at low concentrations [79]. Some cell lines do not take up PLL conjugates while some are sensitive to PLL toxicity, notably the lymphoid cell lines. Interestingly, the administration of PLL-ON conjugates in ternary complexes with polyanions such as heparin reduces the toxic effect of PLL and potentiates the biological effect of the conjugates [81,82]. PLL binds non-specifically to various cell types due to its lack of cellular specificity. To provide proper cellular

targeting, PLL has been conjugated to various cell-specific ligands and antibodies. Details of these studies are described below.

4.2.3. Other methods of antisense delivery

Other ON modifications reported to increase cellular uptake include the attachment of hydrophobic molecules, such as cholesterol and phospholipids, to the ONs [17,83,84]. Coupling of a single cholesterol moiety to an ON appears to increase its intracellular uptake by up to 15-fold [84]. Similarly, anti-HIV cholesteryl-conjugated ONs are more effective than their unmodified counterparts [83]. Similarly, conjugation of an ON to phospholipids was shown to promote its anti-tumor activity [17]. It is not known from these studies whether endocytosis is involved in the uptake of modified ONs.

Avidin, a cationic protein known to be internalized via an adsorptive endocytosis process, has been coupled to ONs [85]. Association of a biotin-conjugated ON with avidin is rapid and of high affinity. Cellular uptake of an avidin-biotin-ON complex was shown to be 4-fold more efficient than the biotin-ON conjugate alone [85]. Finally, direct penetration of ONs into cells has been reported when delivered with streptolysin O, a pore-forming agent [87], or Sendai virus-derived liposomes [77,78].

4.3. Cellular targeting via receptor-mediated endocytosis

The exploitation of the unique specificity of antisense ONs can only be realized if the molecules can be targeted to appropriate cells. Most nucleic acid-based drug targeting systems have been developed for transfecting genes to specific cells [97–104]. Most of these systems utilize receptor-mediated endocytosis as a means to achieve gene targeting. Targeting of hepatocytes via the asialoglycoprotein receptor has been demonstrated both in vitro and in vivo [97,98,105]. Transferrin-mediated gene transfer has also been reported in a variety of cell types [100–102,106]. More recently, gene delivery systems targeting folic acid receptor of tumor cells [107], and Fc receptor of macrophages [108] have been reported.

The utilization of receptor-mediated endocytosis for drug targeting has also been extended to antisense ONs (see summary in Table 3). *c-myb* antisense ONs complexed with transferrin-PLL [111] or folic acid-PLL [115] were found to be more effective in inhibiting HL-60 cell proliferation than unmodified antisense ONs. Furthermore, a phosphorothioate ON complementary to the polyadenylation signal of hepatitis B virus was complexed with asialoorosomucoid-PLL [110]. An increased uptake of the ON and reduced expression of virus surface antigen from virus-transfected Hep G2 cells was observed. Along the same line, antisense ONs targeted to cancer cells via EGF receptor [114] or to macrophages via mannose receptor [112,113] were found to be taken up more efficiently than unmodified ONs.

Drug delivery systems utilizing receptor-mediated endocytosis provide an effective means to achieve drug targeting as well as enhanced drug uptake. The latter is due to the highly efficient nature of the process. For example, the transport protein transferrin may be internalized at a rate of many thousand molecules per second in some

Table 3
Receptor-mediated antisense oligonucleotide delivery

Ligand	Receptor	Target cell	Reference
Asialoorosomucoid	Asialoglycoprotein	171-CAT cells	109
		HepG2 cells	110
Transferrin	Transferrin	HL-60 cells	111
Mannosylated protein	Mannose	J774E cells	112
		Macrophages	113
6-Phosphomannose-BSA	6-Phosphomannose	J774E cells	112
Epidermal growth factor	Epidermal growth factor	Caco-2 cells	114
Folic acid	Folic acid	HL-60 cells	115

cell populations [116,117]. Despite this advantage, in practice, the use of receptor-mediated endocytosis as drug targeting strategies has been limited. This has been largely due to the fact that most drugs lack specific mechanisms to escape from endocytic vesicles once being internalized. Efforts to overcome this problem have been extensive and significant progress has been made (see below).

5. Intracellular fate of antisense oligonucleotides

Although there is strong evidence suggesting that the initial step of ON uptake involves an endocytosis process, subsequent steps by which ONs are transported to the cytoplasm or nucleus are less clear. The intracellular fate of endocytic vesicles containing ONs would depend on the fate of the particular surface receptor to which the ON is associated. Some receptors recycle through acidic compartments in the cytoplasm, releasing their contents and returning to the cell surface undegraded, while others fuse with lysosomes and are eventually degraded. ON recycling following cellular internalization has been reported [59]. Although it is not known whether the recycled ON is structurally intact or degraded, this data suggests that at least some of the intracellular ON pool is exchangable with the extracellular ON. The fact that ONs given exogenously exhibit biological activities implies that some amount of these compounds must exit the endosomes at some point in time during their cycle and reach their targets, although the exit mechanisms have not been elucidated. The observation that certain endosome-disruptive agents can dramatically improve the biologic activity of ONs (see below) also suggests that such escape mechanisms are rather ineffective. Therefore, the ability to facilitate endosomal exit of ONs is an important consideration for their utilization as therapeutic agents.

The fate of ONs once escaped from the endosomal compartments remains controversial, although accumulating evidence seems to suggest that cytoplasmic ONs migrate rapidly to the nucleus. Chin et al. [118] and Leonetti et al. [51] found that ONs microinjected directly into the cell cytoplasm, hence by passing endocytic localization, rapidly diffused into the nucleus. Intracellular localization of ONs was also found to be dependent on the ON backbone structure. Phosphodiester and, to a lesser extent, phosphothioate ONs localized in association with small nuclear ribonucleoproteins (snRNPs), whereas methylphosphonate ONs localized with genomic DNA [118]. Translocation and localization of ONs occured independent of ON sequence, chain length, and cell type used [51,118]. In contrast to these findings, Cerruzzi et al. [119] reported that phosphorothioate ONs localized mainly in the cytosol and relatively small amounts were found in the nucleus or bound to the plasma membrane. A significant portion of the cytoplasmic ON was found to be degraded while those associated with the nucleus or plasma membrane were largely intact. Similar results were obtained with phosphodiester ONs, although degradation was found to be more pronounced in all cell fractions as compared to the phosphorothioate analogs [119]. Work on the intracellular fate of ONs by different investigators is summarized in Table 4.

The discrepancies among results obtained by different groups have not yet been resolved but could possibly be due to a number of factors including the difference in the type of probe labelling, the influence of the probe molecules on ON distribution, and the presence of ON binding molecules in certain compartments. Since ONs were observed in both cytosolic and nuclear compartments, it seems likely that ONs directed against antisense targets in either compartment should be operative. In fact, there are several studies which indicate antisense activities of ONs when their targets are located either in the nucleus or cytoplasm. For example, an antiviral activity was reported with ON targeted to the vesicular stomatitis virus, a virus whose replication cycle is restricted to the cytoplasm [16]. The relationship between the fate of ONs and their antisense activity remains to be elucidated. Understanding such relationship may aid in the design and delivery of more efficient antisense agents.

Table 4
Cellular distribution of antisense oligonucleotides

Cell system	Oligonucleotide	Distribution	Reference
CV-1 epithelial cells	Phosphodiester/ phosphorothioate/ methylphosphonate	Nucleus	118
HeLa cells	Phosphodiester	Nucleus	120
HeLa/CEF cells	Phosphodiester	Nucleus	13
HL-60 cells	Phophodiester	Cytoplasm	59
	Phosphorothioate	Cytoplasm	54
H9/V79 cells	Phosphorothioate	Nucleus	65
L929 fibroblasts	Phosphodiester	Cytoplasm/nucleus	60
MCF-7 cells	Phosphorothioate	Cytoplasm	121
Molt-3 cells	Phosphodiester	Nucleus	122

6. Enhanced cytoplasmic delivery by endosome-disruptive agents

As stated earlier, one of the major limiting factors in effective antisense delivery is the exit of ONs from endosomes. In order to facilitate uptake and cytoplasmic delivery of ONs, several delivery systems have been investigated. Most of these systems rely on an endocytic uptake mechanism. Only a few systems including cationic liposomes [123,124] and Sendai virus-derived liposomes [77,78] seem to allow a direct penetration of ONs through the external cell membrane.

Many opportunistic viruses and toxins possess efficient means to gain entry into the cytoplasm of their target cells [125–131]. Several researchers took advantage of this property for drug delivery purposes. Successful exploitation of this strategy has been reported for gene delivery utilizing inactivated viruses [132–135], fusogenic peptides [134,136,137], and other membrane destabilizing peptides derived from viral envelop proteins [138]. In the case of fusogenic peptides, significant progress has been made in delivering antisense ONs both in vitro and in animal models [78,139]. The use of fusogenic peptides in gene and antisense delivery may provide an attractive alternative to virus particles due to their simplicity, ease of production, and reduced risk compared to the use of viruses.

The study of membrane fusion and disruption processes that occur during viral entry has led to an increased understanding of the role and property of the fusogenic peptides. These peptides generally span a region of about 30 amino acid sequence on the viral envelop protein and contain an alternating pattern of hydrophobic domains interrupted by hydrophilic domains. Under appropriate conditions, most of these peptides undergo conformational change to form amphipathic α-helical structures that can interact with lipid membranes. In the case of several viruses that enter cells via endocytosis, this conformational change is triggered by the low pH within the endosomes [140,141]. One well-studied fusogenic peptide is the trimeric influenza virus hemagglutinin (HA). Endosomal acidification triggers a structural change which exposes the fusion domain located at the amino terminus of the subunit HA-2 [126,142]. The fusion domain contains a membrane-active peptide sequence which at neutral pH, due to repulsion of negatively charged acidic side chains, is prevented from adopting an α-helical structure. In the endosome, however, these carboxyl groups are neutralized by protonation, allowing a transition to a α-helical structure which is able to interact with the endosomal membrane. Such interaction is thought to facilitate the fusion of viral and endosomal membrane, allowing leakage of the viral contents into the cytoplasm of the host cells. Synthetic peptides containing 16–20 amino acids of influenza HA-2 amino terminus have been reported to fuse liposomes and cause leakage of liposomal contents at a pH of less than 6 [143,144].

7. Concluding remarks

Significant progress has been made in attempts to transport antisense ONs into cells using a variety of approaches. Most of these efforts have focused on methods designed to either penetrate the cytoplasmic membrane or to release endocytosed material into the cytoplasm. At present, it is unclear how ONs, once they have gained enter to the cell, migrate to specific intracellular target sites. Most studies to date appear to suggest that ONs in the cytoplasm migrate rapidly to the nucleus. This suggests that ONs directed against antisense targets in the nucleus may be more effective than those aimed at the cytoplasm. However, there are also reports which indicate effective antisense activity against targets located in the cytoplasm. For example, antisense activity has been observed with ONs targeted to the cytoplasmic vesicular stomatitis virus [16]. The relationship between ON activities and their intracellular fate remains to be established.

The next challenge to antisense technology is in its application in humans. Like any other new technology, the development of the antisense concept is still faced with several obstacles. Nonetheless, there is good reason for enthusiastic hope. Indeed, several ON drugs have already demonstrated enough promise to justify clinical trials. They are being tested in patients suffering from leukemia, AIDS, and other diseases in which improved treatments are necessary. It is expected that in the future these ON drugs will be commonly used to treat those diseases for which no effective therapies yet exist.

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Biologic and therapeutic significance of *MYB* expression in human melanoma

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ABSTRACT **We investigated the therapeutic potential of employing antisense oligodeoxynucleotides to target the disruption of *MYB*, a gene which has been postulated to play a pathogenetic role in cutaneous melanoma. We found that *MYB* was expressed at low levels in several human melanoma cell lines. Also, growth of representative lines *in vitro* was inhibited in a dose- and sequence-dependent manner by targeting the *MYB* gene with unmodified or phosphorothioate-modified antisense oligodeoxynucleotides. Inhibition of cell growth correlated with specific decrease of *MYB* mRNA. In SCID mice bearing human melanoma tumors, infusion of *MYB* antisense transiently suppressed *MYB* gene expression but effected long-term growth suppression of transplanted tumor cells. Toxicity of the oligodeoxynucleotides was minimal in mice, even when targeted to the murine *Myb* gene. These results suggest that the *MYB* gene may play an important, though undefined, role in the growth of at least some human melanomas. Inhibition of *MYB* expression might be of use in the treatment of this disease.**

Cutaneous melanoma is a highly malignant and increasingly common neoplasm (1). Because metastatic melanoma remains incurable, new treatment approaches are needed. The ability to selectively disrupt the function of genes involved in malignant cell growth is an increasingly attractive therapeutic strategy. Technologies relevant to this purpose include homologous recombination, which actually destroys the targeted gene (2), and use of reverse complementary (antisense) RNA (3) or DNA (4–6) to interfere with utilization of the target gene's mRNA. The latter may be particularly well suited to therapeutic purposes since antisense oligodeoxynucleotides (ODNs) can be chemically synthesized and introduced directly into cells without the need for viral vectors. Problems attendent to the use and manufacture of viral vectors are thereby avoided. Further, the transient effect of antisense DNA on gene expression, as opposed to permanent alteration of the genome, may be desirable under these circumstances.

We previously reported that phosphorothioate-modified antisense ODNs (antisense [S]ODNs) targeted to the *MYB* protooncogene controlled the growth of a human leukemia in a SCID mouse model (7). *MYB* encodes a transcriptionally active nuclear binding protein, MYB, which plays an important regulatory role in cell proliferation (8) and differentiation (9). *MYB* is located on chromosome 6q22–23 in humans (10), where some human melanomas also have structural aberrations. Altered *MYB* expression has been implicated in the pathogenesis of melanoma (11–14). Accordingly, we targeted the *MYB* gene in human melanoma cells with antisense ODNs to learn more about the biologic importance of its expression in this disease and the therapeutic potential of disrupting its function.

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MATERIALS AND METHODS

ODNs. Unmodified phosphodiester ODNs and [S]ODNs (Lynx Therapeutics, Hayward, CA) complementary to the *MYB* gene were synthesized as reported (8, 15).

Cell Culture and *in Vitro* ODN Exposure. Melanoma cell lines were obtained from the American Type Culture Collection (Hs294T, A375, C32) or from Dupont Guerry (University of Pennsylvania School of Medicine) (SK-MEL-37, WM39). Cells were cultured in 96-well plates (10^3 cells/200 μl per well) to which ODNs (10–100 μg/ml) were added once or on two or five consecutive days. Effects on cell proliferation were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (CellTiter 96 nonradioactive cell-proliferation assay, Promega) (16).

Mice. Seven- to 8-week-old female CB-17/ACRTAC/*scid*/SDS mice were obtained from Taconic Farms. Animals with elevated IgM levels, as measured by ELISA (17), were excluded from the study.

Melanoma Transplantation into SCID Mice and Administration of [S]ODNs. Single-cell suspensions of Hs294T human melanoma cells (2×10^6 in 0.2 ml of Dulbecco's modified Eagle's medium) were injected subcutaneously in the right lower dorsal region of mice. [S]ODNs were administered by subcutaneously implanted Alzet constant-infusion pumps (Alza) (7).

Evaluation of Tumor Growth in Mice. Tumor weights *in vivo* were estimated from three separate diagonal measurements (18). Actual tumor weights were obtained at the time of sacrifice. Statistical significance of tumor weight differences was assessed by the Mann–Whitney nonparametric method.

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction (RT–PCR). *MYB* and β-actin mRNA transcripts were detected by RT–PCR as described save that RT was primed with random hexamers (2 ng/μl) (19). The *MYB* amplification primers corresponded to mRNA nt 195–215 (5′ primer) and nt 444–464 (3′ primer) (10). The PCR product was detected with a probe corresponding to nt 355–375. β-Actin expression was detected with 5′ and 3′ primers corresponding to nt 600–621 and 905–885, respectively. The β-actin PCR product was detected with a ^{32}P-labeled probe corresponding to nt 795–815 (20).

Detection of Antisense [S]ODN in Tumor Tissue. Excised tumors were minced and multiply washed. DNA was extracted by standard methods, and 50 μg was electrophoresed in a 4% low-melting agarose gel (FMC) and then transferred to a nylon membrane (Pall) in 3 M NaCl/0.3 M sodium citrate, pH 7. DNA was probed with a ^{32}P-end-labeled *MYB* sense oligomer complementary to the antisense DNA sequence.

Abbreviations: ODN, oligodeoxynucleotide; [S]ODN, phosphorothioate ODN; RT, reverse transcription.

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RESULTS

***MYB* Expression in Human Melanoma Cell Lines.** Though the integrity of the *MYB* locus on chromosome 6 has been scrutinized (11, 13, 14, 21), data concerning *MYB* expression in melanomas are sparse (22, 23). We therefore screened five human melanoma cell lines (Hs294T, SK-MEL-37, A375, C32, and WM39) for *MYB* mRNA by Northern analysis (19). Total RNA from each cell line (20 μg) was blotted to nitrocellulose and then probed with a ^{32}P-labeled human *MYB* cDNA (24). None of the lines gave a positive signal with this technique. However, when a sensitive RT–PCR was employed, *MYB* mRNA was unambiguously detected in all five cell lines (data not shown). Accordingly, *MYB* is most likely expressed in some melanoma cell lines, albeit at a low level. In accord with this finding, MYB protein levels were below detection limits in Western blot analysis of protein extracted from 7×10^3 SK-MEL-37 and Hs294T cells (data not shown).

Effect of Disrupting *MYB* Expression in Human Melanoma Cells. To determine the biological significance of low-level *MYB* expression in melanoma cells, we targeted the *MYB* message in SK-MEL-37 and Hs294T cells with unmodified or phosphorothioate antisense ODNs. Control DNA sequences were evaluated simultaneously to ensure that any effects observed were sequence specific. In Hs294T cells, for example, exposure to *MYB* sense sequences had no statistically significant effect on cell proliferation in comparison to untreated controls. In contrast, the *MYB* antisense DNA inhibited growth in a dose-responsive manner up to ≈60% ($P <$ 0.001) of control cell values (data not shown). Growth inhibition was accompanied by loss of RT–PCR-detectable *MYB* mRNA, but not β-actin mRNA, suggesting that growth inhibition was secondary to pertubation of *MYB* expression.

Visual examination of the cultures revealed some clue regarding the mechanism of inhibition, which appeared to vary with the cell line. For example, after exposure to *MYB* antisense, Hs294T cells appeared to undergo cytolysis, suggesting that *MYB* perturbation could be a lethal event in these cells (Fig. 1*A*), whereas SK-MEL-37 cells appeared to undergo growth arrest with or without what appeared morphologically to represent differentiation toward a more mature phenotype (Fig. 1*B*).

Relationship of ODN Dose, Frequency of Exposure, and Inhibition of Cell Growth. We also examined cell growth inhibition as a function of ODN concentration and frequency of exposure. When SK-MEL-37 cells were exposed to ODNs, the most important factor for achieving growth inhibition was initial exposure to high concentrations of ODN (Fig. 2). For example, no effect on cell growth was observed when the ODNs were added to cultures in divided doses of 20 μg/ml per day for 2 days, or 10 μg/ml per day for 5 days. In contrast, when cells were exposed to a single bolus of 50 μg/ml, cell growth was inhibited ≈25% in comparison to untreated controls. This relationship was even more apparent at higher doses. A single total ODN dose of 100 μg/ml inhibited growth much more significantly than the same total dose delivered in divided doses of 20 μg/ml per day for 5 days (Fig. 2*A*). Even at doses up to 250 μg/ml, 50 μg/ml per day for 5 days was not as effective as a total of 200 μg/ml given as 100 μg/ml per day for 2 days (50% vs. 70% inhibition, respectively).

To determine whether these results were influenced by possible degradation of unmodified ODN, we carried out similar experiments with Hs294T cells exposed to the more stable [S]ODN. A similar but less strict relationship between extracellular concentration and inhibition of cell growth was again observed (Fig. 2*B*). That is, initial high concentrations were more effective than equivalent final concentrations built up by cumulative lower doses. Accordingly, it appears that for either type of compound, sufficient cellular uptake to inhibit the target gene is achieved only by initial exposure to some critical "high" concentration of compound. Why cumulative lower doses of a stable compound are less effective is uncertain, but examination of this phenomenon may provide valuable information on oligomer uptake mechanisms, intracellular trafficking, and interactions with target mRNA.

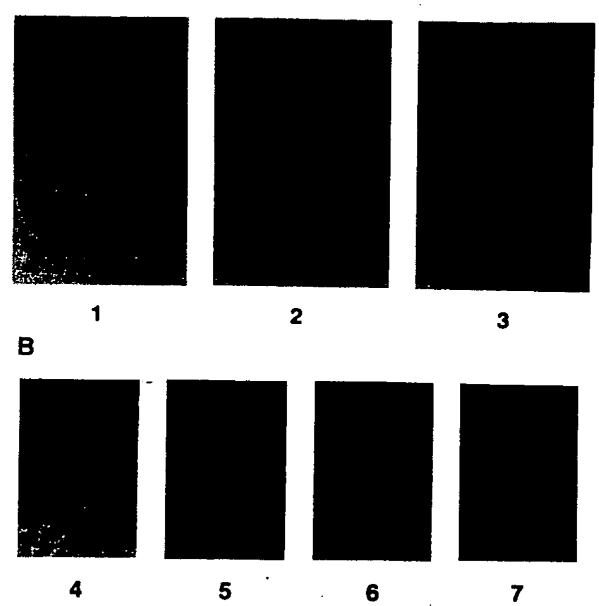

FIG. 1. Photomicrographs of Hs294T and SK-MEL-37 human melanoma cells incubated with *MYB* ODNs (100 μg/ml per day for 5 days). (*A*) Hs294T cells incubated with sense (*1*) or antisense (*2*) [S]ODNs or without ODNs (*3*). (*B*) SK-MEL-37 cells incubated with *MYB* sense (*4*) or antisense (*5*) ODNs without treatment (*6*), or with the differentiation-inducing agent mitomycin C (50 ng/ml per day for 5 days) (*7*). Note the morphologic similarity of cells in *5* and *7*. Under each culture condition cells display a flattened, stellate appearance, multiple dendrites, and increased numbers of pigment granule, all characteristics which suggest that the cells have undergone differentiation (25).

Effect of *MYB* Antisense [S]ODN on Melanoma Tumor Growth *in Vivo*. We examined the effect of the *MYB* antisense

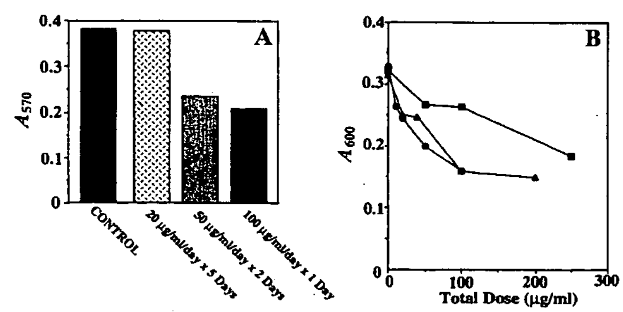

FIG. 2. Effects of concentration and frequency of ODN exposure on melanoma cell growth. (*A*) Effect of a total ODN dose of 100 μg/ml on SK-MEL-37 cell growth. Cells were exposed to *MYB* antisense ODNs for five consecutive days (20 μg/ml per day), two consecutive days (50 μg/ml per day), or once (100 μg/ml). Cell growth was quantitated by MTT assay (A_{570} or A_{600}) 10 days after the start of culture. (*B*) A similar experiment carried out with [S]ODNs on Hs294T cells. Total [S]ODN dose to which the cells were exposed is shown on the abscissa. The total dose was delivered either as a single dose (●) or in equal divided daily doses given over 2 days (▲) or 5 days (■).

DNA on human melanoma cell growth in a SCID mouse model. In the first of three experiments to assess this question, 41 mice were inoculated with Hs294T cells. When tumor nodules became palpable, animals were randomly assigned to receive no treatment (13 animals) or 7-day infusions (500 μg/day; 25 μg/g of body weight) of *MYB* sense (14 animals) or antisense (14 animals) [S]ODN. Animals were examined daily for 40 days to determine the effects of the [S]ODN on survival and tumor growth. The antisense [S]ODN treatment significantly inhibited local tumor growth in comparison to the untreated and sense [S]ODN-treated groups. In fact, until ≈day 35, calculated tumor weights in the antisense group were ≈50% lower than the other groups. After this time, growth in the antisense-treated group recovered and essentially paralleled that seen in the untreated and sense-treated animals. Nevertheless, when the mice were sacrificed on day 40, tumors removed from the antisense-treated animals were significantly smaller ($P < 0.05$), 2.5 ± 0.5 g (mean ± SD), than those from the untreated and sense-treated groups, 3.5 ± 1.7 g and 3.3 ± 1.2 g, respectively.

We then examined the growth-inhibitory effects of the *MYB* antisense [S]ODN against a subclinical tumor burden. In this experiment, mice were subcutaneously inoculated with 2 × 10^6 Hs294T tumor cells. Three days later animals were randomized to receive no treatment (9 mice) or a 7-day infusion (500 μg/day) of *MYB* sense (8 mice) or antisense (10 mice) [S]ODN. Tumor growth was evaluated over a 65-day period (Fig. 3*A*). While no untreated mice were lost during this period, 3 sense- and 4 antisense-treated mice died of uncertain causes. In the remaining animals, inhibition of tumor growth in the antisense-treated group was again noted throughout the observation period and appeared to be greater than that observed in the first experiment. When the mice were sacrificed at 60 days after the pumps were implanted, mean tumor weights of untreated, sense, and antisense groups were 4.5 ± 1.7 g, 4.0 ± 1.5 g, and 2.1 ± 1.2 g, respectively. The differences between these groups were statistically significant ($P < 0.05$). Fig. 4 illustrates typical tumors observed in these mice.

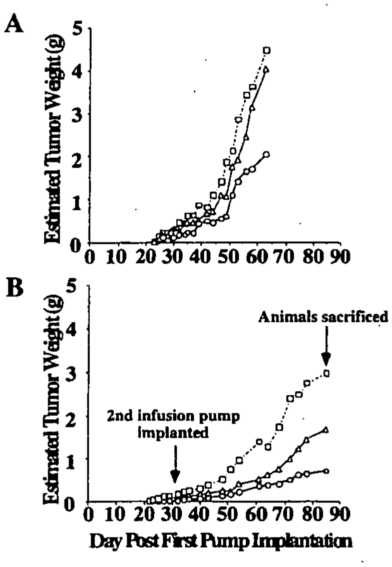

FIG. 3. Effect of *MYB* [S]ODN infusion on human melanoma tumor growth in SCID mice. Doses were 500 μg/day for 7 days initiated 3 days after melanoma cell inoculation (*A*) or 500 μg/day for 14 days administered 3 days after inoculation and then again 16 days after the first infusion ended. ○, Antisense-treated; △, sense-treated; □, untreated.

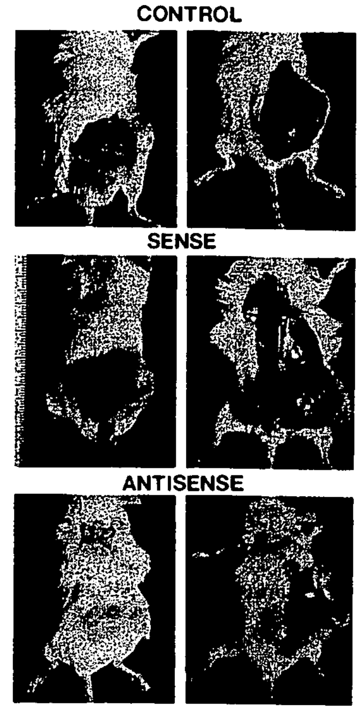

FIG. 4. Representative photomicrographs of melanomas *in situ* in animals which received *MYB* ODN infusions (500 μg/day for 7 days) beginning 3 days after tumor cell inoculation. Infusion pumps can be seen in the sense- and antisense-treated mice.

We lastly examined the effect of a repeat infusion on tumor growth. Mice (10 per group) were again inoculated with 2 × 10^6 tumor cells. Three days later they were randomized to receive no treatment or an infusion of sense or antisense [S]ODN (500 μg/day for 14 days). Sixteen days after the first infusion ended, a repeat infusion of identical dose and duration was begun. In this experiment, 3 control animals died tumor-related deaths during the observation period. In the antisense-treated animals tumor growth inhibition was more dramatic than in the previous experiments and persisted throughout the observation period (Fig. 3*B*). When animals were sacrificed 85 days after the first pump was implanted, mean tumor weights of control (n = 7), sense (n = 9), and antisense (n = 10) groups were 3.0 ± 2.0 g, 1.7 ± 1.5 g, and 0.7 ± 0.5 g. The difference in tumor weights between the untreated and antisense-treated groups was highly significant ($P < 0.01$), as was the difference between the sense-treated and antisense-treated groups ($P < 0.05$). Though it appeared that tumor sizes differed between the untreated and sense-treated groups, the differences were not of statistical significance ($P > 0.05$).

In contrast to the experiments carried out with a lower total dose of [S]ODN, none of the animals in the high dose, repeat-infusion sense- or antisense-treated groups died before the experiment was terminated. These results suggest that [S]ODN toxicity was not a cause of animal deaths.

[S]ODN Uptake in Tumor Tissue and Correlation of Growth Inhibition with *MYB* mRNA Levels. To determine the extent of [S]ODN uptake into tumor tissue and to correlate effects on *MYB* expression with tumor growth, five mice with established tumors (≈0.5 g) were infused with *MYB* antisense [S]ODN (500 μg/day) for 7 days. On days 7, 9, and 11 after the infusion was begun, an animal was sacrificed and its tumor was excised to determine *MYB* mRNA levels in the tissue. *MYB* mRNA was measurably decreased (as normal-

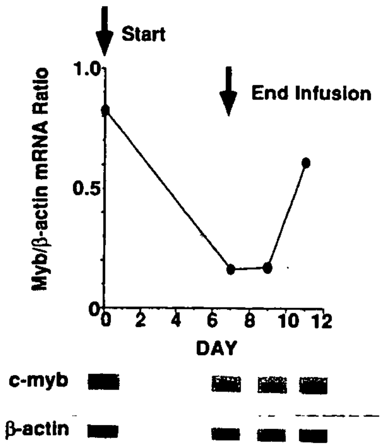

FIG. 5. Effect of *MYB* [S]ODNs on *MYB* mRNA expression in tumor tissue. *MYB* [S]ODNs (500 μg/day for 7 days) were infused into mice with established tumors (≈1.0 g). On days 7, 9, and 11 tumors were excised and total RNA was extracted from approximately equivalent amounts of tissue for RT-PCR detection of *MYB* and β-actin mRNA expression. The relative amount of *MYB* mRNA in each sample was estimated by normalization to the β-actin mRNA detected in the same sample.

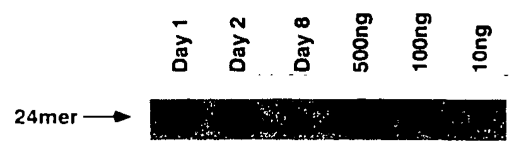

FIG. 6. Detection of *MYB* antisense ODN in tumor tissue. Mice with established tumors were infused with *MYB* antisense ODN (500 μg/day for 7 days). The tumors were excised on days 1, 2, and 8 after start of the infusion. Tumor DNA was extracted, electrophoresed in 4% agarose, and blotted to nylon membranes as noted above. Tumor [S]ODN content was estimated by probing the blotted material with a ^{32}P-end-labeled *MYB* sense oligomer. Relative band intensity was then compared with known DNA standards of 500, 100, and 10 ng (shown in the three lanes at right).

ized to β-actin mRNA), but not completely eliminated, in comparison to control expression (Fig. 5). This decrement in *MYB* expression persisted for ≈2 days after the infusion finished but returned toward baseline thereafter.

Normalization of *MYB* expression may have been related to [S]ODN concentration in tissue falling below a critical level. In support of this hypothesis, [S]ODN levels in the tumor tissue decreased rapidly from an estimated 500 ng (per 50 μg of extracted DNA) during the infusion to 10–50 ng (per 50 μg of extracted DNA) on day 8, 1 day after the infusion finished (Fig. 6). Nevertheless, while human *MYB* expression will be selectively suppressed by the antisense DNA, the PCR primers employed for detection will amplify both human *MYB* mRNA and murine Myb mRNA. Since murine blood and stromal elements gradually infiltrate the tumor, some of the *MYB* mRNA detected could be contributed from this source.

Toxicity of Murine *MYB* Antisense Oligomers in Mice. Mice receiving human *MYB* ODN behaved and fed normally and lost no weight. In addition, histopathologic examination of tissue fixed at the time of sacrifice revealed no organ damage (data not shown). As mentioned above, however, a small number of animals died of unexplained causes during the infusion studies. Such deaths occurred approximately equally in each treatment group and were therefore unrelated to the ODN sequence infused. Further, since there were no unexplained deaths in the animals that received the highest ODN doses (i.e., two infusions), it appears that while [S]ODN toxicity cannot be ruled out as a cause of death in these cases, this explanation seems unlikely.

To model potential toxicity in a human host, mice were also infused with murine *MYB* sense and antisense sequences, up to 1 mg/day for 14 days. No clinically significant untoward effects were observed in these mice (Table 1). The mice behaved normally and their body weights did not change. Of interest, however, mice did manifest thrombocytopenia which appeared to be neither sequence nor dose related. The level of thrombocytopenia was mild to moderate and caused no detectable bleeding abnormality. Animals receiving the highest dose of antisense DNA also manifested splenomegaly. Histopathology of hypertrophied spleens demonstrated lymphoid and myeloid hyperplasia with increased megakaryocytes. We therefore posit that the spleen, an important hematopoietic organ in the mouse, may have been compensating for hematopoietic suppressive effects of the *MYB* antisense [S]ODN.

DISCUSSION

Though the *MYB* gene has been postulated to play a role in the pathogenesis of malignant melanoma, data supporting this hypothesis have been scant and largely inferential (11–14). Using antisense DNA, we now provide some direct evidence that *MYB* gene expression is important for the growth and maintenance of several human melanoma cell lines. Why MYB deprivation causes cytolysis in some cells (Hs294T) and apparent differentiation in others (SK-MEL-37) is unclear. Also enigmatic is the prolonged growth suppression (>2 months) associated with the transient suppression of *MYB*. In the first *in vivo* experiment this might have been more apparent than real, since after an initial delay, growth of the tumor mass in antisense-treated animals appeared to parallel that seen in the control groups. However, this did not seem to be the case in the second (Fig. 4*A*) and third (Fig. 4*B*) *in vivo* experiments, where tumor growth was considerably suppressed. Here one could postulate that

Table 1. Toxicity of murine *MYB* antisense [S]ODNs for BALB/c mice

[S]ODN	Dose, μg/day	Leukocytes, no. × 10^{-3} per mm^3			Hematocrit, %			Platelets, no. × 10^{-3} per mm^3			Spleen weight, mg
		Day 0	Day 14	Day 21	Day 0	Day 14	Day 21	Day 0	Day 14	Day 21	Day 21
Sense	100	3.2	7.7	3.2	53	52	52	554	635	215	140
	300	3.4	6.7	2.9	52	51	51	642	619	218	123
	1000	2.7	3.5	3.6	52	49	51	491	421	346	111
Antisense	100	2.6	4.5	2.6	53	47	48	740	412	188	165
	300	2.1	5.1	2.7	52	44	48	595	397	183	181
	1000	2.7	3.3	4.8	52	44	49	503	230	279	273

[S]ODNs corresponding to murine *Myb* mRNA codons 2–9 were administered to BALB/c mice at various doses for 14 days by Alzet pumps. Blood cells were counted in a hemocytometer and hematocrit was determined by centrifugation of heparinized blood on days 0, 14, and 21. Spleens were weighed immediately after death.

deprivation of MYB led to a persistent, though apparently nonlethal, growth-deprived state. Alternatively, MYB deprivation may have led to cell death in a sensitive subset of cells and to cytostasis in another which eventually recovered. Therefore, the protein probably exerts different functions within and between cells of a given type, and these functions most likely depend on the state of differentiation and cell cycle status. Unmasking these functions through inhibition studies of this type may provide useful clues to the function of MYB in nonhematopoietic cells and to the identity of potential protein partners in these effects.

That we obtained growth inhibition by targeting a gene expressed at such low levels may be viewed as surprising. However, recent reports suggest that low-level gene expression can have significant biological import (26, 27). For example, Burk *et al.* (27) reported that MYB protein cooperatively interacted with C/EBP transcription factor proteins, but only when *MYB* was expressed at a low level. High-level *MYB* expression abrogated this synergy. Accordingly, it is reasonable to hypothesize from all these studies that low-level *MYB* expression is of biological significance in melanoma cells.

The experiments reported herein serve as a paradigm of ODN-based therapeutics for human malignancies. Nevertheless, while it is clear that *MYB* is an effective target in human melanoma, it is not necessarily the best target for this strategy. It is equally straightforward that further development of the antisense strategy will be needed before the successful application of this technique in the clinic can be anticipated. Knowledge concerning DNA uptake mechanisms, intracellular ODN trafficking, mRNA disruption mechanisms, and, of equal importance, how apparent resistance develops will all contribute significantly to the effective pharmaceutical use of these compounds. Accordingly, while this area remains in its scientific infancy, these *in vivo* studies and those of our colleagues (28–30) convince us that modulation of gene expression with antisense DNA is a therapeutic strategy worth pursuing.

The editorial assistance of E. R. Bien is gratefully acknowledged. This work was supported in part by U.S. Public Health Service Grants CA54384 and CA51083 (A.M.G.) and CA25874 (M.H.). A.M.G. is the recipient of a Research Career Development Award from the National Cancer Institute.

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DOCKET NO.: PMB9658 (ISIS-4502)

PATENT

DECLARATION ACCOMPANYING REPLY
FILED UNDER EXPEDITED PROCEDURE
PURSUANT TO 37 C.F.R. § 1.129

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Tullis

Confirmation No.: 9155

Serial No.: 08/078,768

Group Art Unit: 1631

Filing Date: June 16, 1993

Examiner: J. Martinell

For: Oligonucleotide Therapeutic Agent And Methods Of Making Same

EXPRESS MAIL LABEL NO: EV 160093356 US

Box AF
Assistant Commissioner for Patents
Washington DC 20231

Sir:

DECLARATION OF DR. SIDNEY M. HECHT
PURSUANT TO 37 CFR § 1.132

I, Dr. Sidney M. Hecht, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my knowledge are true and statements made on information or belief are believed to be true. The Exhibits attached hereto are incorporated herein by reference.

2. I am the J.W. Mallet Professor of Chemistry and Professor of Biology at the University of Virginia. I serve as Chairman of the Scientific Advisory Board of Orchid BioSciences, as a member of the Scientific Advisory Boards of Xenogen, Galileo Laboratories and Palumed, and as a consultant for Isis Pharmaceuticals. I am President of Pinnacle Pharmaceuticals and a member of the Board of Directors. I also am a member of the Board of Directors of Orchid BioSciences. I serve as an Associate Editor of the *Journal of the American Chemical Society* and sit on the Editorial Advisory Boards of *Anti-Cancer Drug Design*, *Bioconjugate Chemistry* and *Current Medicinal Chemistry-Anticancer Agents*.

From 1981 to 1987 I held concurrent appointments at Smith Kline & French Laboratories, first as Vice President Preclinical R&D, then as Vice President Chemical R&D. I have been an Alfred P. Sloan Fellow and a John Simon Guggenheim Fellow at the Max Planck Institut für Experimentelle Medizin at Göttingen. In 1991 I served as a Professor Associé at the Muséum National d'Histoire Naturelle in Paris and Gastprofessor at the Eidgenössische Technische Hochschule in Zürich; I studied at the Museum again for six months during 2000. I have held numerous lectureships at other universities. I received the 1996 Cope Scholar Award of the American Chemical Society and was selected as Virginia's Outstanding Scientist for 1996. More recently I received the 1998 Research Achievement Award of the American Society of Pharmacognosy.

A copy of my curriculum vitae is attached hereto as Exhibit 1.

3. As early as 1969, I studied mechanisms of protein synthesis via gene expression and regulation thereof. As early as 1972, I co-authored scientific journal articles regarding these studies. Further I have studied the chemistry and biochemistry of nucleic acids since 1966.

My decades of experience as a biological chemist have instilled in me a knowledge of mechanisms of expression of specific genes.

4. I have read and am familiar with the contents of the above-referenced patent application. I have read and agree with the declarations of Dr. Jerry L. Ruth, Dr. Dennis H. Schwartz, and Dr. Stanley T. Crooke previously submitted in connection with the present application. I further understand that the nature of the rejection at issue in the pending application is that the Examiner believes that the pending claims are overbroad in view of the skepticism of critics of antisense technology between October 1981 and the present. It is asserted by the Examiner that the time lapse between October 1981 and the dates of publication of the numerous references cited by the Applicant to support his claim that the methods of the invention work as set forth in the application weighs heavily against the assertion that the instant application provides sufficient guidance to one of skill in the art to practice the claimed invention as early as October 1981. The purpose of this declaration is to address this issue.

I will explain that the concerns of the Examiner stemming from a review of articles by antisense critics including Gura, Rojanasakul, and Hijiya are directed to the immediate clinical applicability of antisense applications rather than to the efficacy of *in vivo* antisense technology applications and, in any event, that those concerns have been proven baseless. I will explain why a significant delay in the reporting of clinical results is routine in the field of drug discovery and development. I also will explain that the numerous clinical investigations conducted on *in vivo* antisense methodologies demonstrate the confidence of pharmaceutical companies and, hence, those skilled scientists who comprise them in the use of antisense technology *in vivo* as detailed by the present application.

In short, the opinions of naysayers that have been lodged against the validity of antisense technology were wrong when made and have been proven to be wrong. Antisense technology works *in vivo* in accordance with the principles of the present invention. The invention as set forth in the application and as presently claimed has been proven to work time and again in the years following the effective filing date of the present application. No further disclosure than that made by Applicant in 1981 was necessary to practice the invention as presently claimed. Applicant was absolutely correct in 1981 and has been proven correct repeatedly thereafter. Additionally, a significant delay in the reporting of clinical results is routine in the field of drug discovery and development, particularly in the present case, where antisense technology was developed by small pioneer companies. The large investments by pharmaceutical companies in the development of antisense technologies underscores their belief in the efficacy of *in vivo* antisense applications. Their continued investments and positive results prove the continued vitality of that belief.

5. Any concerns raised by Gura (*Science*, 270: 575-577 (1995)), Rojanasakul (*Adv. Drug Delivery Revs.*, 18: 115-131 (1996)), and Hijiya (*PNAS USA*, 91:4499-4503 (1994)) are directed toward the immediate clinical applicability of *in vivo* use of antisense technology. These authors do not question the efficacy of antisense applications *in vivo*. For example, Rojanasakul at page 118 queries "Can antisense work in living systems?" and responds by stating that while "there are studies which indicate the ***relative safety*** of antisense [oligonucleotides] *in vivo* . . . ***non-specific side effects*** of [antisense oligonucleotides] have also been reported in mice." Rojanasakul goes on to say that these safety concerns "do not diminish the potential use of [antisense oligonucleotides] *in vivo*, and there are few examples of successful *in vivo* treatment in the absence of specialized delivery systems." *Id.*

Rojanasakul continues, stating that "[c]onsidering the various obstacles that the antisense [oligonucleotides] must encounter prior to their action . . . ***the desired activity of [antisense oligonucleotides] is observed.***" *Id.* (emphasis added).

Gura, a non-research-performing reporter, avers that "some experts in the field . . . argue that clinical trials have begun far too soon." Gura at 575. Such concerns regarding the clinical safety of antisense oligonucleotides were elicited by the side effects detected in some animal studies. For example, Gura describes one set of experiments in which lethality in monkeys administered a one-time, high-dose injection occurred as well as another set of experiments in which a transient decrease in two kinds of white blood cells and changes in heart rate and blood pressure resulted from the high dose administered. *See id.* at 576.

Similarly, the assertion that Hijiya characterizes the field of antisense as being "in its scientific infancy" is misplaced. Hijiya makes clear that antisense oligonucleotides worked therein: "The experiments reported herein serve as a paradigm of [oligodeoxynucleotide]-based therapeutics for human malignancies." Hijiya at 4503. Hijiya reasons that, although *MYB* (a gene) is an effective target of antisense oligonucleotides in human melanoma, "further development of the antisense strategy will be needed before the successful application of this technique ***in the clinic*** can be anticipated." *Id.*

"No drug is free of toxic effects." *See* Fingl and Dixon (Chapter One, "General Principles", In THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 4th edition, L.S. Goodman and A. Gilman, Eds. (1970)) (Exhibit 2). This fact has been known for many years and is as true today as it was when first presented in this textbook. For some authors, to question the clinical safety of a new drug paradigm is not surprising. If raising such questions were to bar patentability of new drugs, there would be no new drugs. Accordingly, some toxic effects of

antisense therapeutics are to be expected. Some expected toxic effects, however, are not an indication that antisense therapeutics do not work *in vivo*.

Moreover, any concerns voiced by Gura, Rojanasakul, and Hijiya regarding the use of antisense technology *in vivo* have been proven to be wrong. The successes achieved in the field of antisense technology have been witnessed, thereby ratifying the views of proponents of antisense at the time of the invention and silencing, indeed converting, many critics to what is clearly the correct view: antisense works *in vivo* as taught by the present application.

A number of articles that corroborate the *in vivo* success of antisense technology have been cited during prosecution of the present application. Further submitted with the accompanying reply is Mirabelli et al. (*Anti-Cancer Drug Design*, 6:647-661 (1991)) (Exhibit 3) which notes that antisense oligonucleotides have demonstrated activities against a broad array of targets, that "the therapeutic indexes of phosphorothioate oligonucleotides appear to be quite high," and that "certain phosphorothioates . . . are extremely well tolerated in animals." Mirabelli at 651. Mirabelli also provides evidence of successful *in vivo* trials of antisense oligonucleotides. *See, e.g.*, Mirabelli at 653.

Crooke (Annu. Rev. Pharmcol. Toxicol., 1992, 32:329-76) (Exhibit 4) corroborates the *in vivo* stability of antisense oligonucleotides, noting that nuclease activity of sera derived from different species varies, with human being the least active. *See, e.g.*, Crooke 1992 at 337. Additionally, modified oligonucleotides enter cells at pharmacologically relevant concentrations. *See id.* at 338-339. *In vivo* pharmacokinetic studies reveal that antisense oligonucleotides are rapidly and broadly distributed following administration in mice, rabbits, and rats. *See id.* at 342-343. Toxicity studies reveal that phosphorothioate oligonucleotides,

for example, have high therapeutic indices and exhibit toxicity only at concentrations far in excess of concentrations at which therapeutic activity is observed. *See id.* at 344; 346-347.

Cossum (*J. Pharm. and Exp. Ther.*, 267(3):1181-1190 (1993)) (Exhibit 5) describes several *in vivo* studies in which phosphorothioate oligonucleotides were shown to be widely distributed following *in vivo* administration in nothing more than phosphate buffer at physiologic pH. *See, e.g.*, Cossum at 1181-1182, 1186. Additionally, Cossum acknowledges that the dosages at which non-antisense effects occur are significantly greater than those at which antisense effects are observed. *See id.* at 1181.

Stepkowski et al. (*J. Immunol.*, 153:5336-5346 (1994)) (Exhibit 6) demonstrates specific inhibition of intercellular adhesion molecule-1 (ICAM-1) expression by antisense molecule IP-3082, thereby promoting heart allograft survival. *See* Stepkowski et al. at 5338. Extension of *in vitro* studies to *in vivo* analyses confirmed the correlation between the efficacy of antisense technology in a Petri dish and in a living organism.

Indeed, a search of the art of "antisense" in the PubMed database reveals approximately 16,986 references demonstrating the extensive interest of the scientific community in the technology of the presently claimed invention (Exhibit 7).

6. The Examiner asserts that the time lapse between the effective filing date of the present application and the numerous references cited in support of enablement of the solicited claims weighs heavily against the claim that the instant application provides sufficient guidance to one of skill in the art to practice the claimed invention as early as the effective filing date of the instant application. I disagree. Had the pharmaceutical industry in 1981 immediately applied its existing knowledge of medicinal chemistry and pharmacology to the teachings of Applicant, I believe that it would have practiced the present invention.

Various factors contributed to this lag, not the least of which include establishment, within an organization, of an internal "champion" for a new technology paradigm where the champion is willing to sponsor and defend reallocation of resources from existing programs to new a program. Also, once acceptance of the new paradigm is made, established pharmaceutical practice requires pharmacologists to perform substantial and numerous pre-clinical studies to determine the toxicological profile, pharmacokinetics, and pharmacodynamics of any potential drug. Thus, my extensive experience as a biological and medicinal chemist have taught me that it is not unexpected that the generation and reporting of pre-clinical and clinical studies by the pharmaceutical industry related to the efficacy of a potential drug does not immediately follow the publication of the first few positive *in vitro* results.

It has been observed by Fingl and Dixon (*see supra*, paragraph 5) that "[n]o drug is free of toxic effects." They further state, however, that "adverse effects do not arise solely because of the inherent toxicity of drugs and the limitations of the methods for early detection of this toxicity. *Many of the adverse effects could be avoided if drugs were used more carefully and more wisely.*" *Id.* at 26 (emphasis added). Further, "[t]he development and evaluation of new drugs in the United States is rigidly controlled by federal regulation administered by the Food and Drug Administration. A new drug may not be marketed for general clinical use until it has been subjected to thorough clinical pharmacological studies and until 'substantial evidence' of its efficacy and safety have been obtained from adequate, well-controlled clinical trials conducted by qualified investigators." *Id.* at 29.

Since both positive and negative results must be included in data packages submitted to regulatory agencies, clinical trials are not performed haphazardly with selective omission of negative results. In other words, slapdash animal studies are not conducted for potential

human therapeutic applications because all data collected is subjected to FDA scrutiny. Accordingly, every study is implemented pursuant to highly rigorous standards and carefully planned conditions. Animal tests suitable to regulatory agency submission require established animal colonies and adequate animal care facilities with appropriate veterinary oversight, the development of which is expensive and time-consuming. Accordingly, careful animal experiments do not yield large volumes of publications that appear in the literature quickly. They require systematic studies that may take years to accomplish. In other words, a significant delay in the reporting of pre-clinical or clinical results is entirely routine in the field of drug discovery and development.

While contributing early-published papers related to *in vitro* related research topics, individual academic researchers, who contribute much of the scientific literature, did not exploit and publish, *in vivo* antisense technology. The reasons for this are varied. The very substantial costs of animal studies, resulting from the necessity of numerous controls as well as the stringent regulations imposed by academic institutions and regulatory agencies, preclude most academic researchers from pursuing such studies absent industrial sponsorship. Additionally, the experiments conducted by most academicians are limited in scope to their existing, well-delineated areas of research interest. Accordingly, academic researchers do not perform isolated experiments that have no bearing on that research interest. Rather, academics are selective in choosing the focus of their experiments, limiting their experimental objectives to the particular area of research that fits into the grand scheme of the research to which their careers are dedicated, for which they have received institutional approval to study, and for which they have been granted funding.

7. The numerous clinical investigations conducted and patents sought on *in vivo* antisense methodologies demonstrate the confidence of pharmaceutical companies and, hence, the skilled scientists that comprise them in the use of antisense technology *in vivo* as detailed by the present application. "Big" pharmaceutical companies became interested in antisense technology after the small pioneer companies confirmed its validity. For example, pioneer companies Hybridon Inc. and Isis Pharmaceuticals, Inc. were incorporated in 1989 for the purpose of developing antisense therapeutics. Gilead Sciences, Inc. formed in 1987 for the same purpose. Genta Inc. was established as a spin-off of Gen-Probe in 1988 with a business objective of developing antisense therapies initiated in Gen-Probe's diagnostic antisense studies. In the mid- and late 1990s, newcomers MethylGene Inc., Inex Pharmaceuticals Corp., and NeoPharma, to name only a few, joined the early-stage companies in exploiting the therapeutic aspects of antisense technology.

In short, antisense technology was developed by small, early stage companies having limited resources. In view of the need of such companies to conserve their limited resources and the knowledge of such companies that a single poorly planned trial yielding a negative outcome could devastate an entire business venture, the pioneer companies in the antisense field had every incentive to perform animal trials carefully and systematically. They conducted animal trials in a highly methodical manner and at timepoints dictated by scientific and business judgment to advance to that phase in the process of moving their drug candidates toward IND status. Pharmaceutical companies including Isis Pharmaceuticals, Genta Inc. and Hybridon Inc. and their present or past large pharma partners including Novartis, Lilly, Abbott, Merck, Aventis, Amgen, Roche and Boehringer Ingelheim have invested huge amounts of time and money to verify the efficacy of antisense drugs in an

effort to propel them through clinical phases and into the market. Given the enormous costs associated with drug development and marketing, pharmaceutical companies would not have invested so heavily in the development of antisense technologies if they believed antisense molecules would not work *in vivo*.

8. In summary, the opinions of naysayers that have been lodged against the validity of antisense technology were wrong when made and have been proven to be wrong. Antisense technology works *in vivo* in accordance with the principles of the present invention. The invention as set forth in the application and as presently claimed has been proven to work time and again in the years following the effective filing date of the present application. No further disclosure than that made by Applicant in 1981 was necessary to practice the invention as presently claimed. Applicant was absolutely correct in 1981 and has been proven correct repeatedly thereafter. Additionally, a significant delay in the reporting of clinical results is routine in the field of drug discovery and development, particularly in the present case, where antisense technology was developed by small pioneer companies. The large investments by pharmaceutical companies in the development of antisense technologies underscores their belief in the efficacy of *in vivo* antisense applications. Their continued investments and positive results prove the continued vitality of that belief.

Date: 03/04/03

Dr. Sidney M. Hecht

Attachment
Exhibits 1, 2, 3, 4, 5, 6 and 7

THE PHARMACOLOGICAL BASIS OF THERAPEUTICS

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The Pharmacological Basis of Therapeutics

FOURTH EDITION

A TEXTBOOK OF
PHARMACOLOGY, TOXICOLOGY, AND
THERAPEUTICS FOR
PHYSICIANS AND MEDICAL STUDENTS

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In this textbook, reference to proprietary names of drugs is ordinarily made only in chapter sections dealing with preparations. Such names are given in SMALL-CAP TYPE, usually immediately following the official or nonproprietary titles. Proprietary names of drugs also appear in the Index.

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SECTION

I

Introduction

CHAPTER

1 GENERAL PRINCIPLES

Edward Fingl and Dixon M. Woodbury

The basic pharmacological concepts summarized in this chapter apply to the characterization, evaluation, and comparison of all drugs. A clear understanding of these principles will facilitate subsequent study of the individual drugs. Many of these topics have been more extensively discussed in the textbook by Goldstein and coworkers (1968) and in the symposium edited by Tedeschi and Tedeschi (1968).

SCOPE OF PHARMACOLOGY

In its entirety, *pharmacology* embraces the knowledge of the history, source, physical and chemical properties, compounding, biochemical and physiological effects, mechanisms of action, absorption, distribution, biotransformation and excretion, and therapeutic and other uses of drugs. Since a *drug* is broadly defined as any chemical agent that affects living processes, the subject of pharmacology is obviously quite extensive.

For the physician and the medical student, however, the scope of pharmacology is less expansive than indicated by the above definitions. The clinician is interested primarily in drugs that are useful in the prevention, diagnosis, and treatment of human disease, or in the prevention of pregnancy. His study of the pharmacology of these drugs can be reasonably limited to those aspects that provide the basis for their rational clinical use. Secondarily, the physician is also concerned with chemical agents that are not used in therapy but are commonly responsible for household poisoning as well as environmental pollution. His study of these substances is justifiably restricted to the general principles of prevention, recognition, and treatment of such toxicity or pollution. Finally, all physicians feel a sense of responsibility toward the growing sociological problem of the abuse of drugs.

A brief consideration of its major subject areas will further clarify how the study of pharmacology is best approached from the standpoint of the specific requirements and interests of the medical student and practitioner. At one time, it was essential for the physician to have a broad botanical knowledge, since most drugs used in therapy were obtained from plants, and since the physician had to select the proper plants from which to prepare his own crude medicinal preparations. However, relatively few drugs are still obtained from natural sources, and most of these are highly purified or standardized and differ little from synthetic chemicals. Hence, the interests of the modern clinician in *pharmacognosy* are correspondingly limited. Nevertheless, scientific curiosity should stimulate the physician to learn something of the *sources* of drugs, and this knowledge often proves practically useful as well as interesting. He will find the *history* of drugs of similar value.

The preparing, compounding, and dispensing of medicines at one time lay within the province of the physician, but this work is now delegated almost completely to the pharmacist. However, to write intelligent prescription orders, the physician must have some knowledge of the *physical and chemical properties* of drugs and their available

dosage forms, and he must have a basic familiarity with the *practice of pharmacy*. When the physician shirks his responsibility in this regard, he invariably fails to translate his knowledge of pharmacology and medicine into prescription orders and medication best suited for the individual patient. The few details essential to the writing of correct prescription orders are summarized in the Appendix.

The study of the biochemical and physiological *effects* of drugs and their *mechanisms of action* is termed *pharmacodynamics*. It is an experimental medical science that dates back only to the latter half of the nineteenth century. As a border science, pharmacodynamics borrows freely from both the subject matter and the experimental technics of physiology, biochemistry, microbiology, and pathology. It is unique mainly in that attention is focused on the characteristics of drugs. As the name implies, the subject is a dynamic one. The student who attempts merely to memorize the pharmacodynamic properties of drugs is foregoing one of the best opportunities for correlating the entire field of preclinical medicine. For example, the actions and effects of the saluretic agents can be fully understood only in terms of the basic principles of renal physiology and of the pathogenesis of edema. Conversely, no greater insight into normal and abnormal renal physiology can be gained than by the study of the pharmacodynamics of the saluretic agents.

Pharmacodynamics also deals with the *absorption, distribution, biotransformation*, and *excretion* of drugs. These factors, coupled with dosage, determine the concentration of a drug at its sites of action and, hence, both the intensity and the time course of its effects. Many basic principles of biochemistry and enzymology and the physical and chemical principles that govern the active and passive transfer and the distribution of substances across biological membranes are readily applied to the understanding of this important aspect of pharmacodynamics.

Another ramification of pharmacodynamics is the correlation of the actions and effects of drugs with their chemical structures. Such *structure-activity relationships* are an integral link in the analysis of drug action, and exploitation of these relationships among established therapeutic agents has often led to the development of better drugs. However, the correlation of biological activity with chemical structure is usually of interest to the physician only when it provides the basis for summarizing other pharmacological information.

The physician is understandably interested mainly in the effects of drugs in man. This emphasis on *human pharmacology* is justified, since the effects of drugs are often characterized by significant interspecies variation, and since they may be further modified by disease. In addition, some drug effects, such as those on mood and behavior, can be adequately studied only in man. However, the pharmacological evaluation of drugs in man may be limited for technical and ethical reasons, and the choice of drugs must be based in part on their pharmacological evaluation in animals. Consequently, some knowledge of *animal pharmacology* and *comparative pharmacology* is helpful in deciding the extent to which claims for a drug based upon studies in animals can be reasonably extrapolated to man.

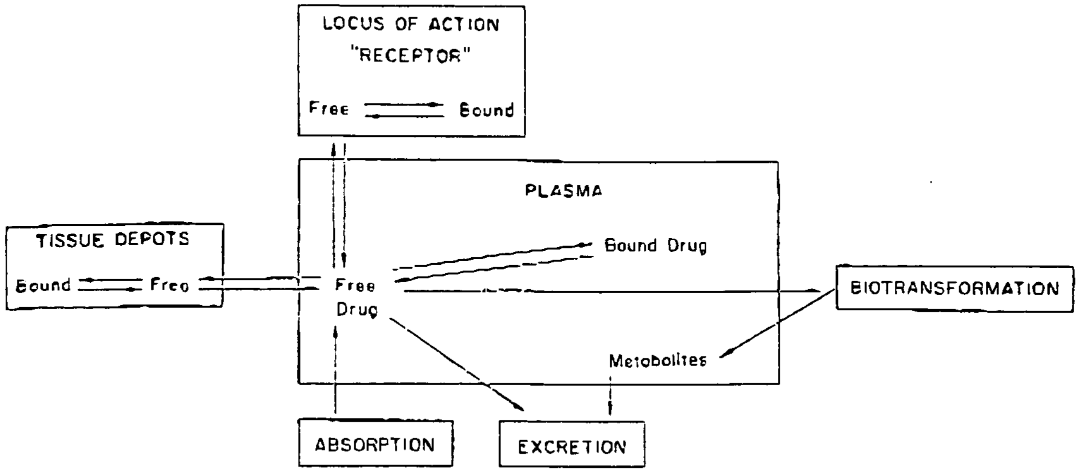
Pharmacotherapeutics deals with the *use* of drugs in the prevention and treatment of disease. Many drugs stimulate or depress biochemical or physiological function in man in a sufficiently reproducible manner to provide relief of symptoms or, ideally, favorably to alter the course of disease. Drugs of this type are designated *pharmacodynamic agents*. Other drugs, known as *chemotherapeutic agents*, are useful in therapy because they have only minimal effects on man but can destroy or eliminate parasites. Whether a drug is useful for therapy is crucially dependent upon its ability to produce its desired effects with only tolerable undesired effects. Thus, from the standpoint of the physician interested in the therapeutic uses of a drug, the *selectivity* of its effects is one of its most important characteristics.

In the medical curriculum, pharmacotherapeutics is often neglected in the initial teaching of pharmacology, because the formal course is usually taught during the preclinical years, and it is thought that the student lacks the necessary clinical background. This is unfortunate, since drug therapy is rationally based upon the correlation of the actions and effects of drugs with the physiological, biochemical, and microbiological aspects of disease. Pharmacodynamics provides one of the best opportunities for this correlation during the study of both the basic and the clinical medical sciences.

Toxicology is that aspect of pharmacology that deals with the adverse effects of drugs. It is concerned not only with drugs used in therapy but also with the many other chemicals that may be responsible for household, environmental, or industrial intoxication. The toxic effects of the pharmacodynamic and chemotherapeutic agents are properly considered an integral part of their total pharmacology. The toxic effects of other chemicals is such an extensive subject that the physician must usually confine his attention to the general principles applicable to the prevention, recognition, and treatment of drug poisonings of any cause.

ABSORPTION, DISTRIBUTION, BIOTRANSFORMATION, AND EXCRETION

To produce its characteristic effects, a drug must achieve adequate concentrations at its sites of action. Although obviously a function of the amount of drug administered, the concentrations attained also depend upon the extent and rate of its absorption, distribution, binding or localization in tissues, inactivation, and excretion. These factors are depicted in the following scheme:



PHYSICOCHEMICAL FACTORS IN TRANSFER OF DRUGS ACROSS MEMBRANES

Either directly or indirectly, the absorption, distribution, biotransformation, and excretion of a drug all involve its passage across cell membranes. It is essential, therefore, to consider the mechanisms by which drugs cross membranes and the physicochemical properties of molecules and membranes that influence this transfer. Important characteristics of a drug are its molecular size and shape; degree of dissociation or ionization of an acid, base, or salt; and relative lipid solubility of its charged and uncharged forms. Single cells and cell nuclei have a simple plasma membrane. In contrast, the intestinal epithelial boundary is represented by a single cell layer, and the skin barrier is composed of several layers of cells. Despite these anatomical differences, the diffusion and transport of drugs across these various boundaries are remarkably similar.

Cell Membranes. The classical observations by Overton and by Collander and Bärlund led to the theory that the cell (plasma) membrane consists of a thin layer of lipoid material interspersed with minute water-filled channels, often called pores. Subsequent studies have indicated that the plasma membrane consists of a bimolecular lipoid sheet bound on both sides by protein. The thickness of the membranes is of the order of 100 Å. On the basis of electron micrographic studies, two categories of membranes have been distinguished (Sjöstrand, 1967). The first type, exemplified by the plasma membrane, primarily controls the composition of the medium bounded by the membrane, by means of characteristic permeability and transport properties. The second type, exemplified by the mitochondrial membrane, is primarily associated with enzymatic function; the structure of such membranes is more complex and consists of a thicker inner surface thought to contain highly ordered multienzyme systems in the form of globular proteins.

Passive Processes. Drugs move across membranes either by passive transfer processes or by specialized active transfer systems. The membrane is not involved in the passive processes, and the drug molecules penetrate either by passage through aqueous channels in the membrane or by dissolving in the membrane substance. Both nonpolar lipid-soluble compounds and polar substances that possess sufficient lipid solubility move across the predominantly lipoid plasma membrane by *passive diffusion*. Their transfer is directly proportional to the concentration gradient across the membrane and the lipid:water partition coefficient of the drug. The greater the partition coefficient, the higher is the concentration of drug in the membrane and the faster is its diffusion. However, after a steady state is attained, the concentration of the drug is the same on both sides of the membrane. Passage through channels is called *filtration*, since it involves bulk flow of water as a result of a hydrostatic or osmotic difference across the membrane. The bulk flow of water carries with it any water-soluble molecule that is small enough to pass through the channels. Filtration is a common mechanism for transfer of many small, water-soluble, polar and nonpolar substances. The size of the membrane channels differs in the various body membranes. Capillary endothelial cells have large channels (40 Å), and molecules as large as albumin pass from the plasma to the extracellular fluid or from the plasma into the glomerular filtrate. In contrast, the channels in the red-cell membrane, the intestinal epithelium, and most cell

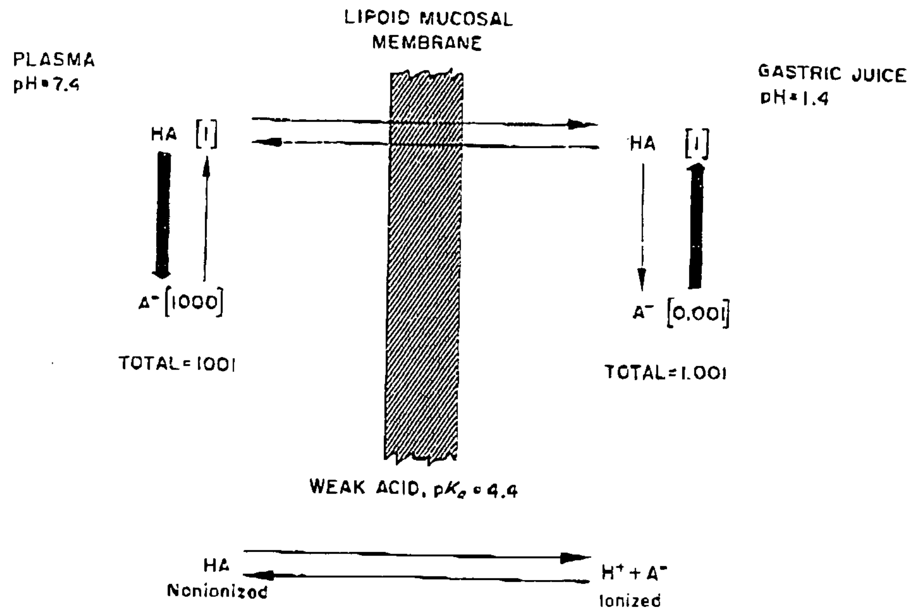


Figure 1–1. *Influence of pH on the distribution of a weak acid between plasma and gastric juice, separated by a lipoid membrane.*

Only the nonionized moiety can readily penetrate the membrane; hence, at equilibrium its concentration is the same in both compartments. The degree of dissociation of the acid on each side depends on the pH of the plasma and gastric juice. The total concentration difference between the two sides is a direct function of the pH gradient across the membrane.

The values in brackets represent relative concentrations of the ionized and nonionized forms on each side of the membrane. The thick vertical arrows point in the direction of the predominant form of the weak acid at the indicated pH.

membranes are about 4 Å in diameter and permit passage only of water, urea, and other small, water-soluble molecules. Such substances generally do not pass through channels in cell membranes if their molecular weights are greater than 100 to 200.

Most inorganic ions are sufficiently small to penetrate the pores in membranes, but their concentration gradient across the cell membrane is generally determined by the transmembrane potential (*e.g.*, chloride ion) or by active transport (*e.g.*, sodium and potassium ions).

Weak Electrolytes and Influence of pH. Most drugs are weak acids or bases and are present in solution as both the nonionized and ionized species. The nonionized portion is usually lipid soluble and can readily diffuse across the cell membrane. In contrast, the ionized fraction is often unable to penetrate the lipoid membrane because of its low lipid solubility, or to traverse the membrane channels because of its size. If the ionized portion of a weak electrolyte can pass through the channels, or through the membrane, it will distribute according to the transmembrane potential. For example, inorganic ions, like chloride, bicarbonate, and bromide, and the ionized form of drugs, like salicylate or 5,5-dimethyl-2,4-oxazolidinedione (DMO), are distributed unequally across the red-blood-cell membrane.

If the ionized portion of a weak electrolyte cannot penetrate the membrane, its distribution will be determined by its pK_a and the pH gradient across the membrane. If a pH gradient exists, the degree of ionization of the electrolyte on the two sides of the membrane will differ. Accordingly, even though the concentration of the nonionized fraction on the two sides is the same, the total concentration of the drug (ionized plus nonionized) on the two sides will also differ. To illustrate the effect of pH on distribution of drugs, the partitioning of a weak acid ($pK_a = 4.4$) between plasma ($pH = 7.4$) and gastric juice ($pH = 1.4$) is depicted in Figure 1–1. It is assumed that the gastric mucosal membrane behaves as a simple lipoid barrier that is permeable only to the lipid-soluble, nondissociated form of the acid. The ratio of nonionized to ionized drug at each pH can be calculated from the Henderson-Hasselbalch equation. Thus, in plasma, the ratio of nonionized to ionized drug is 1:1000; in gastric juice, the ratio is 1:0.001. The total concentration ratio between the plasma and the gastric juice sides of the barrier is therefore 1000:1. For a weak base with a pK_a of 4.4, the ratio is reversed. (For more detailed discussions of drug transfer by passive processes and the effect of pK_a and pH on drug movements, *see* Albert, 1952; Brodie and Hogben, 1957; Schanker, 1962.)

Specialized Active Transport Processes. The

passive processes described above do not explain the passage of all drugs across cell membranes. Specialized active transport processes appear to be responsible for the rapid cellular transfer of certain foreign organic ions and polar molecules, as well as many natural substrates such as sugars, amino acids, and pyrimidines.

Active transport processes differ from passive processes in that they exhibit selectivity, saturability, and a requirement for energy. Specialized active transport is generally thought to be mediated by carriers, membrane components that form a complex with the substance to be transported. The complex is presumed to be formed on one side of the membrane and to diffuse to the other side where the substance is released, after which the carrier returns to the original surface to repeat the process. If the process transports a substance against an electrochemical gradient (uphill transport) and is blocked by metabolic inhibitors, it is called *active transport;* ions such as Na^+, K^+, and I^-, amino acids, certain strong organic acids and bases, and ionized forms of weak electrolytes are transported across the renal tubule, choroid plexus, and liver cells in this manner. Carrier transport that shows selectivity, saturability, and blockade by metabolic inhibitors but in which the substance does not move against a concentration gradient is called *facilitated diffusion.* Glucose, pyrimidines, and some of their antimetabolites are transported across gastrointestinal epithelium and other cell membranes by this process. Transport is facilitated by attachment to a carrier and is more rapid than simple diffusion.

Absorption

It is of practical importance to know the manner in which drugs are absorbed. The rate of absorption largely determines the latent period between administration and onset of action; it is also important in determining dosage. In many instances, absorption influences the choice of the route by which a drug is administered.

Many variables, in addition to the physicochemical factors discussed above, influence the absorption of drugs. Absorption from all sites of administration is dependent upon drug *solubility.* Drugs given in solution are more rapidly absorbed than those given in solid form. For those given in solid form, the rate of dissolution becomes the limiting factor in their absorption. Local conditions at the site of absorption alter solubility. Thus, at the low pH of the gastric juice, many acidic drugs are absorbed slowly because they precipitate in the fluids of the stomach, and dissolution occurs very slowly. Highly insoluble substances, such as barium sulfate, are not absorbed from the alimentary tract at all. The *concentration* of a drug also influences its rate of absorption. Drugs ingested or injected in solutions of high concentration are absorbed more rapidly than are drugs in solutions of low concentration. The *circulation to the site of absorption* also affects drug absorption is most importantly determined area, brought about by massage or local application of heat, enhances absorption of a drug, whereas decreased blood flow, produced by vasoconstrictors, shock, or other factors, slows absorption. The area of the *absorbing surface* to which a drug is exposed is one of the more important determinants of the rate of drug absorption. Obviously, drugs are absorbed very rapidly from large surface areas such as the pulmonary endothelium, peritoneal cavity, and intestinal mucosa, and factors that increase the area over which a drug is spread enhance absorption. Since the absorbing surface is determined largely by the *route of administration,* it is evident that the rate of drug absorption is most importantly determined by this factor. Often there is a choice in the route by which a therapeutic agent may be given, and a knowledge of the advantages and disadvantages of the different methods of administration is then of primary importance.

Alimentary Tract. The oral route is the most ancient method of drug administration. It is also the safest, most convenient, and most economical. Drugs given by mouth may be retained there and absorbed through the oral mucosa, or they may be swallowed and absorbed from the stomach and intestine. Disadvantages to the oral route of ingestion of drugs include emesis as a result of irritation to the gastrointestinal mucosa, destruction of some drugs by the digestive enzymes, formation with food of complexes that cannot be absorbed, and necessity for cooperation on the part of the patient.

Oral Mucosa. The mucosal lining of the mouth behaves as a lipoid barrier to the passage of drugs, and their absorption through the oral mucosa involves the same principles outlined above for most epithelial membranes. Absorption from the oral mucosa is rapid. A higher concentration of the drug in the blood may be achieved than by

absorption lower in the alimentary tract because metabolism of drugs as a result of passage through the liver is avoided, and because the drug is not subjected to possible destruction by the gastrointestinal secretions or to formation of complexes with foods. However, substances that are distasteful or that are irritating should not be given by this route. The sublingual route of administration permits rapid absorption of a variety of drugs. It is a convenient method that is often overlooked.

Gastrointestinal Tract. Absorption of drugs from the gastrointestinal tract is for the most part explainable in terms of pH-dependent, simple nonionic diffusion across the gastrointestinal epithelial membrane, as described above and depicted in Figure 1–1. For example, alcohol, a lipid-soluble nonelectrolyte, is rapidly absorbed into the blood stream by diffusion across the gastric mucosa. Weak acids, such as salicylates and barbiturates, which are predominantly nonionized in the acid gastric contents, are also readily absorbed from the stomach. In contrast, weak bases, such as morphine, quinine, ephedrine, and tolazoline, which are predominantly ionized at the pH of gastric juice, are poorly absorbed through the gastric mucosa and are absorbed mainly through the intestinal mucosa. If the gastric contents are made alkaline, acidic compounds become more ionized and are less well absorbed. Conversely, basic compounds become less ionized and are better absorbed. The pH at the surface of the intestinal epithelium where absorption occurs is about 5.3; consequently, weak bases are more readily absorbed and weak acids less readily absorbed than in the stomach. Increasing the pH of the intestinal contents enhances this difference. Quaternary ammonium compounds, streptomycin, and other completely ionized, lipid-insoluble drugs are very slowly absorbed. Succinylsulfathiazole and other drugs, even the nonionic forms of which are lipid insoluble, are also poorly absorbed from the gastrointestinal tract. The quaternary ammonium compounds may be absorbed by formation of a phosphatido-peptide intermediate.

Theoretically, the concentration ratio of a weak electrolyte between gastric juice and plasma can be as high as 1 millionfold. Experimentally, the value never exceeds 40-fold, because gastric mucosal blood flow limits the rate at which the drug can be supplied to, or removed from, the gastric juice and equilibrium is never attained. The establishment of concentration gradients of weak electrolytes across the gastric mucosa is a purely physical process and does not require an active transport system. All that is necessary is to have a membrane preferentially permeable to the nonionizable form of the weak electrolyte and the establishment of a pH gradient across the membrane. The establishment of the pH gradient is, however, an active transport process requiring expenditure of energy to secrete hydrogen and chloride ions.

Factors other than the rate of passage of drugs across the gastrointestinal mucosa influence the absorption of drugs from the alimentary tract. One of these factors is the rate of *gastric emptying*. Changes in gastric emptying might be expected to have opposite effects on absorption of weak acids and weak bases because of the difference in gastric and intestinal pH. However, because of the large surface area of the intestine, the absorption of most drugs is reduced if gastric emptying is retarded. Other factors that affect absorption, such as *solubility* of a drug in gastrointestinal fluids and *concentration* of the administered solution, have been noted above. Absorption from the alimentary tract may also be retarded or decreased if the ingested drug is unstable in gastrointestinal fluid, or if it becomes bound to food or other gastrointestinal contents. Simultaneous ingestion of food also delays absorption by decreasing gastric emptying.

The rate of absorption of solid forms of drugs is dependent mainly on their disolution rate in gastrointestinal fluids, and this factor is the basis for the oral *prolonged-action* or *sustained-release* pharmaceutical preparations. These preparations include tablets-within-tablets, slowly disintegrating tablets, pellets in capsules, slowly dissolving salts in solid or liquid suspension, and drugs embedded in ion-exchange or inert plastic matrices, all of which are designed to produce slow, uniform absorption of the drug and thereby provide a sustained effect for 8 hours or longer. In addition, they are intended to provide rapid onset of action by immediate release of sufficient active ingredient to raise the level of the drug in the blood to that producing the desired therapeutic effect. Potential advantages of such preparations are reduction in the frequency of administration of the drug as compared with conventional forms, maintenance of a therapeutic effect overnight, and decreased incidence of undesired effects by elimination of the

peaks in drug concentration that often occur after repeated administration of nonsustained-release dosage forms. Sustained-release preparations may also reduce the hazard of defaulting from prescribed treatment by allowing the patient to take the drug less often. In the case of some drugs, such as certain amphetamines, antihistamines, antitussives, and tranquilizers, uniformly effective sustained-release preparations have been achieved and are being used successfully in therapy. However, each drug must be evaluated separately for its suitability as a sustained-release preparation. It is obvious that drugs given for a brief therapeutic effect should not be in the sustained-release form. In addition, not all marketed preparations are reliable. Dissolution rate of some preparations in gastrointestinal fluid may be quite irregular because of technical problems associated with their manufacture and of variations in gastrointestinal pH, gastric emptying, intestinal motility, and other physiological factors that influence drug absorption. Since the total dose of drug ingested at one time is equal to 3 to 4 doses of the conventional form of the drug, release of the entire amount at once due to occasional failure of the material to hold together may occur and lead to toxicity. Also, failure of adequate release may compromise the therapeutic effect. Therefore, it is incumbent on the physician who uses preparations of this type to establish a need for a prolonged-action preparation and also to evaluate its uniformity, reliability, and safety. This is especially necessary since the same drugs made into sustained-release formulations by different processes or by different manufacturers may have release rates and durations of actions that vary considerably from each other. The use of these nonuniform and poorly reliable preparations cannot be justified at the present time.

Rectal Mucosa. Drugs can also be given by the *rectal* route. This is often useful when the oral route is precluded by vomiting or when the patient is unconscious. In addition, the absorbed drug does not pass through the liver before entry into the systemic circulation. However, absorption by this route is often irregular and incomplete, and many drugs cause irritation of the rectal mucosa.

Injection. The injection of medicinals has at times certain distinct advantages over oral administration. In some instances, this route of administration is essential for the drug to be absorbed in active form. Absorption is usually more rapid and more predictable than when a drug is given by mouth. The effective dose can therefore be more accurately selected. In emergency therapy, parenteral administration is particularly serviceable. If a patient is unconscious, uncooperative, or unable to retain anything given by mouth, parenteral therapy may become a necessity. On the other hand, there are several disadvantages to the injection of drugs. Strict asepsis must be maintained in order to avoid infection, an intravascular injection may occur when it is not intended, pain may accompany the injection, and it is often difficult for a patient to perform the injection himself if self-medication is a necessary procedure. Parenteral therapy is also more expensive and less safe than oral medication. Absorption of lipid-soluble drugs from subcutaneous and intramuscular sites occurs by simple nonionic diffusion through the capillary membranes into the blood and is directly proportional to the lipid:water partition coefficient of the drug. The rate of absorption is also influenced by the total area of the absorbing capillary membranes and by the solubility of the substance in the interstitial fluid. Lipid-insoluble drugs are absorbed into the blood by penetration through the relatively large aqueous pores in the endothelial membrane; larger molecules, such as proteins, gain access to the circulation by way of lymphatic channels. Some large molecules and microcrystalline substances are absorbed from these sites by phagocytosis.

Subcutaneous. Injection at this site is often utilized for the administration of medicinals. It can be used only for drugs that are not irritating to tissue; otherwise, a slough may occur. The rate of absorption following subcutaneous injection of a drug is often sufficiently even and slow to provide a fairly sustained effect. Moreover, it may be willfully varied by well-known technics. The subcutaneous injection of a suspension of a drug in a vehicle in which it is insoluble results in a very slow rate of absorption. For example, the rate of absorption of a suspension of insoluble protamine insulin is slow as compared with that of soluble insulin. The incorporation of a vasoconstrictor agent in a solution of a drug to be injected subcutaneously also retards absorption. This principle is utilized in the combination of epinephrine with local anesthetics. Absorption of drugs implanted under the skin in a solid pellet form occurs slowly over a period of weeks or months; several hormones are effectively administered in this manner. (*See* review by Schou, 1961.)

Intramuscular. Drugs are often injected deep into muscle tissue. From this site, drugs in aqueous solution are rapidly absorbed by the diffusion processes described above. It is possible to dissolve or suspend drugs in oil and inject them intramuscu-

larly. This leads to a very slow and even rate of absorption. Drugs with low solubility, such as the repository penicillin preparations, are also slowly absorbed from intramuscular sites. Irritating substances that cannot be injected subcutaneously may be given by the intramuscular route.

Intraperitoneal. The peritoneal cavity offers a large absorbing surface from which drugs enter the circulation rapidly. Intraperitoneal injection is a common laboratory procedure, but it is seldom employed clinically. The dangers of infection and adhesions are too great to warrant the routine use of this route in man.

Intravenous. Drugs in aqueous solution can be introduced directly into the circulation, usually into a vein. The factors concerned in absorption are circumvented by intravenous injection, and the desired blood concentration of a drug is obtained with an accuracy and immediacy not possible by any other procedure. In some instances, as in the induction of surgical anesthesia by a barbiturate, the dose of a drug is not predetermined but is adjusted to the response of the patient. Also, certain irritating and hypertonic solutions can be given only in this manner, for the blood vessel walls are relatively insensitive and the drug, if injected slowly, is greatly diluted by the blood.

On the other hand, there are many dangers that attend intravenous injections. Unfavorable reactions are more prone to occur than when any other route is used. Once the drug is injected there is no retreat, whereas when subcutaneous administration is employed absorption can be stopped by occluding venous return from the area of injection. Repeated intravenous injections are dependent upon the patency of veins. Drugs in an oily vehicle or those that precipitate blood constituents or hemolyze erythrocytes should not be given by this route. Unless specifically indicated, drugs should never be given directly into the blood stream.

Intra-arterial. Occasionally a drug is injected directly into an artery in order to localize its effect in a particular tissue or organ. Antineoplastic agents are sometimes given in this manner for the treatment of localized tumors. This procedure requires great care, and its use should be reserved for experts.

Intrathecal. The blood-brain barrier and the blood–cerebrospinal fluid barrier often preclude or slow the entrance of drugs into the central nervous system (CNS). Therefore, when local and rapid effects of drugs on the meninges or cerebrospinal axis are desired, as in spinal anesthesia or acute CNS infections, drugs are sometimes injected directly into the spinal subarachnoid space.

Pulmonary Endothelium. Gaseous and volatile drugs may be inhaled; they are then absorbed through the pulmonary endothelium or mucous membranes of the respiratory tract and in this way gain rapid access to the circulation. The principles governing absorption and excretion of gases and vapors are discussed in Chapter 5. Also, solutions of drugs can be atomized and the fine droplets in air (aerosol) inhaled. Advantages are the almost instantaneous absorption of a drug into the blood and, in the case of pulmonary disease, local application of the drug at the desired site of action. For example, epinephrine can be given in this manner for the treatment of bronchial asthma. The main disadvantages are poor ability to regulate the dose, cumbersomeness of the methods of administration, and the fact that many gaseous and volatile drugs produce irritation of the pulmonary endothelium.

Mucous Membranes. Absorption of drugs takes place readily through many of the mucous membranes of the body other than those of the alimentary canal, and the more accessible ones are often employed for this purpose. Certain drugs can reach the circulation through the mucous membranes of the vagina, urethra, conjunctiva, nose, and oropharynx. In many instances, drugs are applied at these sites for their local action, but on occasion it is the systemic effect that is desired. In fact, local anesthetics may sometimes be absorbed so rapidly from the mucous membranes that they produce systemic toxicity.

Skin. Few drugs readily penetrate the intact skin. Absorption of those that do is proportional to their lipid solubility since the epidermis behaves as a lipoid barrier; the dermis, however, is freely permeable to many solutes. As a result, toxic effects can sometimes be produced by absorption through the skin of highly lipid-soluble substances (*e.g.*, a lipid-soluble insecticide in an organic solvent). Absorption through the skin can be enhanced by suspending the drug in an oily vehicle and rubbing the resulting preparation into the skin. This method of administration is known as *inunction*. Lipid-insoluble molecules and ions penetrate slowly. The rate of transfer of drugs that ionize, however, can be increased by a procedure known as *iontophore-*

sis. This is accomplished by passage of a galvanic current through a solution of drug applied to the skin underneath an electrode. The procedure is not often used, since large amounts of drugs are required, the dose given is difficult to determine, and the method is cumbersome.

DISTRIBUTION

After a drug is absorbed or injected into the blood stream, it can enter or pass through the various body fluid compartments —plasma, interstitial fluid, transcellular fluids, and cellular fluids. Some drugs cannot pass cell membranes and, therefore, are restricted in their distribution and in their sites of action, whereas others pass through cell membranes and thereby distribute throughout all fluid compartments. In addition, some drugs may accumulate in various areas as a result of binding, dissolving in fat, or active transport. The accumulation may be at the locus of action of the drug or, more often, in some other location. In the latter situation, the site of accumulation may serve as a storage depot for the drug.

Storage Depots. Many areas of the body serve as reservoirs for drugs because selective accumulation occurs at these sites. Stored drug is in equilibrium with that in plasma and is released as plasma concentrations are reduced. As a result, effective plasma levels of the drug are maintained for a longer period, and pharmacological effects are correspondingly prolonged. However, to obtain therapeutically effective levels initially, adequate priming doses must be given to saturate the binding sites. The following are the major drug storage sites in the body.

Plasma Proteins and Other Extracellular Depots. Upon entering the blood, a drug may be bound to plasma proteins. Ordinarily the binding is to albumin. However, other plasma proteins also bind drugs. The binding involves reversible bonds of the ionic, hydrogen, and van der Waals types. The extent of binding depends on the particular drug. For example, it may be high, as with phenylbutazone (98%); or low, as with barbital (5%); or practically nil, as with antipyrine. If affinity of a drug for the albumin is high, the plasma proteins may serve as a rather large storage depot for the drug. For example, suramin, an effective agent in the therapy of trypanosomiasis, is strongly bound to plasma protein, and a single dose of the drug confers protection against infection for 3 months or more.

Some drugs are stored in connective tissue because they are bound to the strongly ionic groups of the mucopolysaccharides. Bone may also be a reservoir for drugs, and substances such as heavy metals and tetracyclines are stored there. The nature of the accumulation in bone is not known, but it probably is due to adsorption of the substances to the bone-crystal surface or incorporation into the crystal lattice.

Cellular Depots of Drugs. Many drugs accumulate in higher concentrations in cells than in the extracellular fluids. If the intracellular concentration is high, the tissue involved may serve as a large storage depot. Accumulation in cells may be brought about by active transport or by binding to tissue constituents. Tissue binding of drugs usually occurs with proteins, phospholipids, or nucleoproteins and is generally reversible. A classical example of a drug that binds to tissue constituents is the antimalarial agent quinacrine. This drug may achieve a concentration in liver 2000 times that of the plasma level 4 hours after a single dose. After chronic administration, the accumulation is even more pronounced and may amount to 22,000 times the plasma level. The binding occurs in the nuclei, probably with nucleoproteins.

Fat as a Depot for Drugs. Many drugs have a high lipid solubility and are stored by physical solution in the neutral fat. In obese persons, the fat content of the body may be as high as 50%, and even in starvation it makes up 10% of body weight; hence, fat can serve as an important depot for storage of lipid-soluble drugs. The majority of a drug with a high lipid:water partition coefficient may enter fat. For example, as much as 70% of the highly lipid-soluble barbiturate thiopental may be present in body fat 3 hours after administration.

Transcellular Depots of Drugs. Drugs also cross epithelial cells into the transcellular fluids and may accumulate in these fluids. The major transcellular depot in the body is the gastrointestinal tract. A drug that is poorly soluble in gastrointestinal fluid will be slowly absorbed and serve as a

reservoir for maintaining the level in the plasma and increasing its duration in the body. Weak bases are passively concentrated in the stomach from the blood, because of the large pH differential between the two fluids; the gut thus serves as a storage depot for such compounds.

Drugs do not generally accumulate in the *cerebrospinal fluid,* because no protein is present for binding, they exit relatively rapidly by way of the arachnoid villi, some are actively transported out of cerebrospinal fluid, and there is only a small pH gradient across the choroid plexus. Other transcellular fluids, such as *aqueous humor, endolymph,* and *joint fluids,* do not generally accumulate drugs and constitute only minor storage depots in the body. The *luminal fluid* of the *thyroid* serves as the major storage depot for iodine in the body and can concentrate drugs such as the perchlorate and some other monovalent anions.

Passage of Drugs into and across Cells. The passage of drugs across cell membranes involves the same factors discussed above for membranes in general. Weak electrolytes penetrate cells by simple diffusion in the nonionized form in proportion to their lipid: water partition coefficient and are distributed between extracellular and intracellular fluids in proportion to the pH difference of the two fluids. However, since the pH difference between intracellular and extracellular fluids is small (7.0 versus 7.4), the concentration gradient across the membrane is also small. Weak bases are concentrated slightly inside of cells, while the concentration of weak acids is slightly lower in the cells than in extracellular fluids. Lowering the pH of extracellular fluid increases the intracellular concentration of weak acids and decreases that of weak bases, provided that the pH change does not simultaneously affect the binding, biotransformation, or excretion of the drug. Elevating the pH produces the opposite effects. Nonelectrolytes enter cells by diffusion and generally in proportion to their lipid solubility, but small molecules such as urea penetrate through aqueous channels in the membrane. Penetration of strong acids and bases that are completely ionized depends upon the permeability of, and the potential difference across, the cell membrane.

The penetration of drugs into subcellular particles such as mitochondria, although little studied, appears to be an important aspect of drug distribution and action. The mitochondrial membrane is lipoid in nature, and penetration of drugs follows the same principles as for cell membranes.

Penetration into Central Nervous System and Cerebrospinal Fluid. The entrance of drugs into the CNS and the cerebrospinal fluid is a special aspect of cellular penetration; however, in general, it follows the same principles as for transfer across other cells. The blood-brain barrier is located not at the surface of brain cells but between the plasma and extracellular space of the brain, at the basement membrane of capillary endothelial cells. Transfer of drugs across neuronal cell membranes is like that across any other cell membrane. The blood–cerebrospinal fluid barrier is located at the choroid plexus. Lipid-insoluble drugs and inorganic and organic ions enter the brain much more slowly than do lipid-soluble substances. Their rate of entrance is proportional to the size of the molecule; large molecules like inulin penetrate slowly, whereas small ions like chloride or small nonelectrolytes like urea penetrate more rapidly, but still slowly as compared to the rate in other tissues. The choroid plexus is also slowly permeable to small ions and lipid-insoluble substances but poorly permeable or impermeable to large lipid-insoluble substances.

The routes of exit of drugs from the cerebrospinal fluid differ from the routes of entrance. All drugs and many endogenous metabolites, regardless of lipid solubility or molecular size (including molecules as large as plasma albumin), leave the cerebrospinal fluid by flow through the arachnoid villi. The speed of exit is the same for all the substances and depends on the rate of the bulk flow of cerebrospinal fluid. In addition, if the drug is lipid soluble, it can exit by the same route it entered, namely, by diffusion across the lipid portions of the blood–cerebrospinal fluid boundary. Drugs can also diffuse into and out of the brain through the blood-brain boundary; exit by this route is aided by bulk flow of cerebrospinal fluid through the brain and into the capillaries. In addition, certain drugs and endogenous metabolites may be removed from the cerebrospinal fluid by specialized, active transport processes, similar to those that exist for organic ions in the renal tubules (*see* below). (For summaries of drug penetration into and out of the CNS, *see* Rall and Zubrod, 1962; Schanker, 1962.)

Placental Transfer of Drugs. A knowledge of the principles of transfer of drugs across the placenta is important since drugs can exert toxic effects on the fetus and may induce congenital anomalies. The transfer occurs primarily by simple diffusion; carrier-mediated transport is generally restricted to endogenous substrates. Nonionized drugs of high fat solubility readily enter the fetal blood from the maternal circulation. Penetration is least with drugs possessing a high

degree of dissociation and/or low lipid solubility. (For summaries of placental transfer of drugs, *see* Schanker, 1962; Symposium, 1964; Goldstein *et al.*, 1968.)

Redistribution. Although termination of drug effect is usually accomplished by biotransformation and excretion, it may also result from redistribution of the drug from its site of action into other tissues; however, it is stored there in an active form and its ultimate elimination from the body is still dependent on biotransformation and excretion. If the inital dose saturates the storage site, a subsequent dose of the drug may produce a prolonged effect. The factors involved in redistribution of drugs have been extensively studied for thiopental and are described in Chapter 9.

BIOTRANSFORMATION

Many drugs are lipid-soluble, weak organic acids and bases that are readily reabsorbed in the renal tubules. To be excreted more rapidly, they must be transformed into more polar compounds. These more ionized, less lipid-soluble metabolites are also less able to bind to plasma and tissue proteins, less likely to be stored in fat depots, and less able to penetrate cell membranes. Thus, this type of biotransformation usually results in inactivation of the drug. Occasionally, however, activation may result, or an active drug may be transformed into a metabolite that is also active. In such cases, termination of action takes place by further biotransformation or by excretion of the active metabolite in the urine.

General Considerations. Although much of the information concerning the biotransformation of drugs is based upon observations in laboratory animals, many clinical studies demonstrate that similar mechanisms occur in man. However, the rates at which the reactions proceed in the various species are often very different; a drug may be rapidly inactivated and have a short duration of action in animals, yet be much more slowly inactivated and have a long duration of action in man.

The chemical reactions concerned in the biotransformation of drugs can be classified as *nonsynthetic* and *synthetic*. The nonsynthetic reactions involve oxidation, reduction, or hydrolysis, and may result in activation, change in activity, or inactivation of the parent drug. The synthetic reactions, also called conjugation, involve coupling between the drug or its metabolite and an endogenous substrate that is usually a carbohydrate, an amino acid, derivatives of these or an inorganic sulfate. Synthetic reactions almost invariably result in inactivation of the parent drug.

Various patterns of biotransformations involving nonsynthetic and synthetic reactions may be distinguished. (For summaries of drug biotransformations, *see* Symposium, 1962; Williams, 1959, 1963; Goldstein *et al.*, 1968; many others.)

One type may be exemplified by the following reactions:

$$\underset{\text{(inactive)}}{\text{Prontosil}} \xrightarrow{\text{reduction}} \underset{\text{(active)}}{\text{Sulfanilamide}} \xrightarrow{\text{acetylation}} \underset{\text{(inactive)}}{\text{Acetylsulfanilamide}}$$

In this case, the inactive parent drug is converted to an active chemotherapeutic agent by a reductive process, and the active drug is subsequently inactivated by a synthetic reaction involving acetylation. The sojourn of the active drug in the body depends upon the relative rates of activation and inactivation.

A second pattern of biotransformation is depicted in the following scheme:

$$\underset{\text{(active)}}{\text{Phenacetin}} \xrightarrow{\text{de-ethylation}} \underset{\text{(active)}}{\text{Acetaminophen}} \xrightarrow{\text{conjugation}} \underset{\text{(inactive)}}{\text{Acetaminophen glucuronide}}$$

This biotransformation involves change of the parent drug to a metabolite that is also active until conjugated. The first step may occur without the synthetic second step. For example, the anticonvulsant trimethadione is converted to the active metabolite 5,5-dimethyl-2,4-oxazolidinedione (DMO) by oxidative N-demethylation. DMO is not further metabolized but is slowly excreted in the urine. A change in the type of biological activity may also occur. For example, aniline, a highly toxic substance with pharmacological effects similar to those of acetanilid and phenacetin, is converted in the body to a metabolite that causes methemoglobin formation.

Inactivation may also occur in both the first and second steps, as follows:

$$\underset{\text{(active)}}{\text{Phenobarbital}} \xrightarrow[\text{hydroxylation of the aromatic ring}]{\text{oxidative}} \underset{\text{(inactive)}}{\text{Hydroxyphenobarbital}} \xrightarrow{\text{conjugation}} \underset{\text{(inactive)}}{\text{Hydroxyphenobarbital ethereal sulfate or glucuronide}}$$

The first phase of inactivation may be oxidative, reductive, or hydrolytic. Reactions of this type are extremely common and are often responsible for the termination of drug activity. The synthetic step may not be present in some cases. For example, procaine is inactivated by hydrolytic cleavage to *p*-aminobenzoic acid and diethylaminoethanol.

Another biotransformation, usually resulting in inactivation, involves only the synthetic reaction. A typical example is the following:

$$\underset{\text{(active)}}{\text{Salicylamide}} \xrightarrow{\text{conjugation}} \underset{\text{(inactive)}}{\text{Salicylamide glucuronide}}$$

These reactions are often called detoxification, and many drugs are inactivated in this manner. Rarely, a change in activity may occur.

Specific Processes. Biotransformation of drugs occurs mainly in the liver, but also takes place in plasma, kidney, and other tissues.

Oxidation. An important group of oxidative enzymes (called mixed-function oxidases) is located in hepatic microsomes. The reactions catalyzed by these oxidative enzymes include N- and O-dealkylation, aromatic ring and side chain hydroxylation, sulfoxide formation, N-oxidation, N-hydroxylation, and deamination of primary and secondary amines. The replacement of a sulfur by an oxygen atom (desulfuration) can also occur. Thus, parathion, which contains a —P=S group and is inactive, is converted to paraoxon, which contains a —P=O group and is an insecticide with anticholinesterase properties. NADPH and molecular oxygen are essential in most of these reactions, as discussed below. Some drug oxidations are mediated by enzymes other than those of the hepatic microsomal system—for example, oxidation of alcohols in the soluble fraction of the liver by *dehydrogenation*. In addition, monoamine and diamine oxidases are mitochondrial enzymes, found especially in liver, kidney, intestine, and nervous tissue, that oxidatively deaminate several naturally occurring amines as well as a number of drugs. Also, many halogenated compounds used as insecticides and industrial solvents are *dehalogenated* in the soluble fraction of the liver. The reactions include replacement of a halide atom by a hydroxyl group, loss of hydrogen halide, and replacement of halide by an acetylcysteine group (mercapturic acid formation).

Reduction. Hepatic microsomes and some other tissues also contain enzymes that catalyze the reduction of nitro groups and the cleavage and reduction of the azo linkage. The reactions are catalyzed by flavoproteins that require NADPH as the hydrogen donor and in some cases cytochrome P-450, as discussed below. Examples are the nitro reduction of chloramphenicol and the azo reduction of PRONTOSIL. The conversion of chloral hydrate to trichloroethanol is a reduction reaction catalyzed by alcohol dehydrogenase.

Hydrolysis. These reactions involve deamidation by hepatic enzymes and de-esterification by esterases located in plasma, soluble fraction of liver, and many other tissues. Examples include the hydrolysis of procaine, procainamide, meperidine, acetylcholine, and many other esters and amides. Peptidases in plasma, erythrocytes, and many other tissues are involved in the biotransformations of the biologically active polypeptides.

Synthesis (Conjugation). A variety of synthetic reactions are involved in the inactivation of drugs. The substances are conjugated to form inactive, highly ionized, water-soluble substances that are readily excreted in the urine. These reactions are termed synthetic, since they are endothermic and require a source of energy, usually adenosinetriphosphate (ATP). The reaction involves activation of either the drug or the conjugating agent. Conjugating enzymes occur mainly in the liver but also in other tissues, particularly the kidney. The major conjugation reactions are: *glucuronide* formation, an important pathway in the metabolism of phenols, alcohols, and carboxylic acids, a process that involves the formation of uridine diphosphate–glucuronic acid (UDPGA), which serves as a donor of glucuronic acid to the drug acceptors and is catalyzed by various transferase enzymes; *ribonucleoside* and *ribonucleotide* synthesis, usually with analogs of purines and pyrimidines to form biologically active anticancer agents, reactions catalyzed by the same enzymes (soluble fraction) responsible for formation of the naturally occurring nucleosides and nucleotides; *sulfate* conjugation, mainly with phenols and involving formation of an active sulfate donor by reaction of inorganic sulfate with ATP; *acetylation*, usually involving the conjugation of acetyl coenzyme A (CoA) with an acceptor amine, such as sulfanilamide; *O-*, *S-*, and *N-methylation*, with certain types of phenolic compounds, nicotinic acid, and epinephrine and norepinephrine, the methyl group being supplied by S-methyladenosylmethionine; and *glycine* conjugation, with aromatic acids such as benzoic and salicylic acids and involving the formation of a CoA-drug intermediate.

Sites and Mechanisms of Biotransformation. Drugs are metabolized by a variety of enzymes involved in intermediary metabolism. For example, some alcohols are inactivated by alcohol dehydrogenase; succinylcholine, by plasma pseudocholinesterase; 6-mercaptopurine, by xanthine oxidase. Administered biogenic amines and their precursors are biotransformed by the enzymes (monoamine oxidase, tyrosine hydroxylase, etc.) involved in the normal synthesis and metabolism of these compounds. However, the majority of drugs are metabolized by the hepatic microsomal enzymes.

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Microsomal Drug-Metabolizing System. The enzyme systems concerned in the biotransformation of many drugs and also of steroid hormones and lipids appear to be located in the hepatic endoplasmic reticulum (ergastoplasm). Fragments of this network are isolated from liver homogenates by various technics in the fraction generally called *microsomes.* The microsomal enzymes catalyze many of the nonsynthetic and synthetic biotransformations described above. Since so many drugs are metabolized by this system, it is important to consider these enzymes in some detail and to note the factors that influence their activity.

The endoplasmic reticulum resembles a kind of pipeline or canal system in the cell and may function in the transport of substances from one area of a cell to another; it is continuous with the cell membrane and with the nuclear membrane. As identified by electron microscopy, the reticulum consists mainly of a membrane that often bears small ribonucleoprotein particles, called *Palade granules*, which cause the reticulum to have a rough surface. Only the smooth-surfaced microsomes are thought to possess enzymes that can metabolize drugs. The rough-surfaced microsomes are concerned with protein synthesis.

Most of the oxidative and some of the reductive drug-metabolizing enzymes in the hepatic microsomes are unusual in that they require NADPH and molecular oxygen for their action. Electron transfer appears to involve three carriers, as depicted in Figure 1–2.

NADPH is oxidized by a flavoprotein enzyme, NADPH–cytochrome C reductase, which in turn appears to be reoxidized by an incompletely characterized enzyme thought to be a non-heme iron (NHI) protein. This reduced carrier then is reoxidized by cytochrome P-450, so named because it absorbs light at 450 $m\mu$ when exposed to carbon monoxide, a property that makes possible its analytical determination. Furthermore, carbon monoxide also blocks the metabolism of many drugs by the hepatic microsomal system. The drug to be metabolized appears to be bound firmly to oxidized P-450. This drug-oxidized P-450 complex, after reduction by the NHI protein, reacts directly with molecular oxygen to form a drug-oxidized P-450-O complex and water. A hydroxyl group is transferred to the drug, and oxidized P-450 is regenerated as the oxidized drug is released.

The rate of drug biotransformation appears to be related not only to the amount of drug–P-450 complex but also to its rate of enzymatic reduction. The amount of cytochrome P-450 in liver varies from species to species, strain to strain, and even individual to individual; this might explain the large variability in rate of metabolism of a particular drug observed in man. When the rate of a drug oxidation is increased by prior treatment with an inducing agent such as phenobarbital (*see* below), the amount of cytochrome P-450 also increases and this can explain the enhancing effect of phenobarbital on drug-oxidation reactions. There is ample spectral evidence to support the concept that drugs bind directly to cytochrome P-450. Apparently, this carrier is necessary, not only for active oxidation of a drug but also for the binding of the microsomes of the compound to be oxidized.

The reduction of some nitro and azo groups by the liver microsomes is also catalyzed by this system; in these reactions, the drug is first oxidized to an intermediate, which is then reduced by the H ions from NADPH. These hepatic enzymes also function for the inactivation of endogenous steroid hormones and the oxidation of fatty acids. As mentioned below, many drugs compete with these steroids, and possibly the fatty acids, for the oxidative enzymes involved. (*See* Symposium, 1965; Gillette, 1966; Conney, 1967; Goldstein *et al.*, 1968; many others.)

Lipid solubility is an important, but not the only, requirement for a drug to be metabolized by the hepatic microsomes since this property favors the penetration of a drug into the endoplasmic reticulum and the binding with cytochrome P-450. Endogenous substrates except steroids and fatty acids are not affected since most are polar, water-soluble compounds. The evolutionary development of the drug-metabolizing system in hepatic microsomes is a fascinating story in comparative pharmacology that is beyond the scope of this chapter. Summaries of this aspect have been presented by Brodie and Maickel in a symposium (1962).

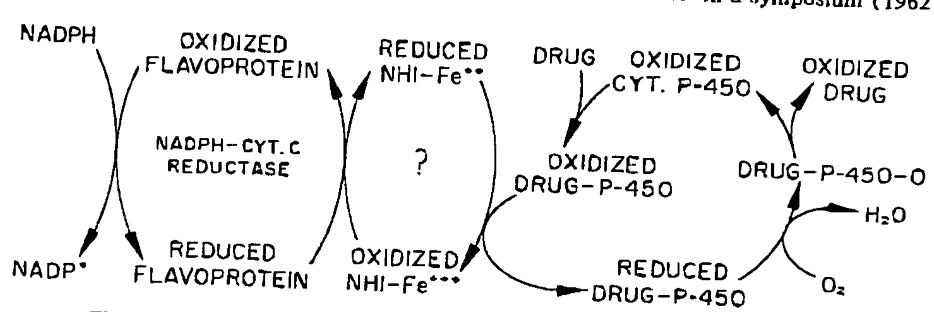

Figure 1–2. *Electron transfer in the microsomal drug-metabolizing system.*

The activity of the drug-metabolizing enzymes in hepatic microsomes, as well as the structure and amount of smooth-surfaced endoplasmic reticulum, is influenced markedly by the administration of various drugs and hormones and by the age, sex, strain, temperature, nutritional status, and pathological state of the animal. Some of these factors are discussed below.

Inhibitors. The effects of many drugs are enhanced and prolonged by interference with their enzymatic destruction by hepatic microsomal enzymes. The first such compound described was SKF 525A (β-diethylaminoethyl-2,2-diphenylpentanoate), a compound with no other appreciable pharmacological effects. Many drugs structurally related to SKF 525A and various other compounds produce similar inhibition of microsomal enzymes.

In man, a number of drugs inhibit the metabolism of other drugs. For example, phenylbutazone and bishydroxycoumarin inhibit the metabolic inactivation of tolbutamide and profound hypoglycemia occurs if one of these drugs is administered concurrently with tolbutamide. Similarly, bishydroxycoumarin and disulfiram inhibit the metabolism of diphenylhydantoin and can cause accumulation of the anticonvulsant to toxic levels. In addition, some agents are known to enhance the anticoagulant effect of bishydroxycoumarin by inhibition of its metabolism; serious hemorrhage can result if care is not taken when these drugs are given concurrently. These inhibitors appear to act in a variety of ways: interference with NADPH-generating systems, inhibition of reduction of P-450, and blockade of the transfer of active oxygen from P-450 to the drug substrate. They also may inhibit by more than one mechanism. For example, SKF 525A is converted to a substance that inactivates P-450, but it also presumably combines with the active site of an N-dealkylase in hepatic microsomes since the inhibition is competitive; it is also a competitive antagonist of microsomal and plasma esterases. (*See* Gillette, 1966.)

Stimulators. Activity of the hepatic microsomal enzymes may also be increased by the administration of drugs. Prior treatment of experimental animals with various agents increases their ability to metabolize both the administered drug as well as other related and unrelated compounds and some endogenous substrates. After prior treatment of rats with phenobarbital, the metabolism of zoxazolamine, hexobarbital, and phenobarbital itself is increased and their pharmacological effects are proportionately reduced. Various carcinogenic hydrocarbons, steroid hormones, and at least 200 other agents exert similar effects, but still others drugs have no such enhancing effect on enzyme activity. The increased activity of the microsomal enzymes is probably due to enhanced synthesis of cytochrome P-450, NADPH–cytochrome C reductase, and other enzymes involved in the metabolism of drugs. This probability is supported by the facts that ethionine, puromycin, and actinomycin, which interfere with protein synthesis, block the effect and that the enhancement of microsomal enzyme activity does not occur when the stimulating agents are added to the enzymes *in vitro*. Also, inducers increase RNA polymerase activity. Enzyme induction by some drugs occurs to a limited extent in hepatic mitochondria and in kidney, gastrointestinal tract, adrenal, lung, and, occasionally, other tissues as well.

The observations that one drug can stimulate its own metabolism, that of another drug, or of normal body constituents have wide implications for chronic toxicity tests, crossover drug studies in animals and man, chronic drug therapy with single or multiple drugs, and the development of tolerance to drugs. In each situation, the pharmacological effects of the second dose or of the second drug administered will be modified by the first, since its metabolism will be altered by stimulation of the drug-metabolizing enzymes.

Drug-induced stimulation of microsomal enzyme activity occurs in man and plays an important role in drug therapy. Enzyme induction is responsible for some altered pharmacological effects that occur during chronic medication. Many examples have been described, but only a few will be presented here to illustrate this important concept. Other examples are discussed throughout the text. (For excellent reviews, also *see* Burns, 1964; Burns and Conney, 1965; Conney, 1967, 1969.) Concurrent administration of phenobarbital and bishydroxycoumarin results in lower plasma levels of bishydroxycoumarin and less anticoagulant effect than when bishydroxycoumarin is administered alone. Even larger doses of the anticoagulant cannot maintain adequate blood levels of the drug in the presence of phenobarbital. If phenobarbital is discontinued, the plasma level and anticoagulant effect of bishydroxycoumarin increase to such an extent that severe bleeding may result. Thus, when

concurrent therapy with a stimulator of drug metabolism and another drug is undertaken, extreme care must be observed when the dose of either is increased or reduced. Phenobarbital, in addition, stimulates the metabolism and lowers the blood levels of diphenylhydantoin, griseofulvin, and aminopyrine. As a result of enzyme induction by drugs, the hydroxylation of androgens, estrogens, progestational steroids, and glucocorticoids, and the conjugation of bilirubin are increased. Chronic administration of a drug may also stimulate its own metabolism and thereby cause tolerance. Environmental carcinogens, insecticides, and cigarette smoking have been shown to induce hepatic drug-metabolizing enzymes in experimental animals. The implications of this effect for man are now being assessed. Cigarette smoking, for example, enhances the metabolism of nicotine in man, and this may explain the tolerance to nicotine that occurs in smokers. Whether smoking also stimulates the metabolism of commonly used drugs in man is an important, but unanswered, question. The inductive effect of phenobarbital on glucuronide conjugation has been used to advantage in the treatment of hyperbilirubinemia in infants with congenital nonhemolytic jaundice; phenobarbital treatment lowers the free plasma bilirubin concentration, and the jaundice disappears. In such infants there is a genetic deficiency in formation of glucuronides, and phenobarbital treatment enhances the activity of glucuronyl transferase that carries out this reaction.

EXCRETION

The kidney is the most important organ for drug excretion. Drugs and their metabolites excreted in the feces are derived either from unabsorbed orally ingested drug or from metabolites excreted in the bile and not reabsorbed from the intestinal tract. Excretion of drugs in milk is important not because of the amounts eliminated but because the excreted drugs are potential sources of unwanted pharmacological effects or toxicity in the nursing infant. Pulmonary excretion is of importance mainly for the elimination of anesthetic gases and vapors, but some metabolites of toxic substances are excreted by this route. Factors affecting pulmonary excretion are discussed in Chapter 5.

Drugs are eliminated from the body either unchanged or as metabolites. Generally, the more polar compounds are excreted unchanged. The less polar, lipid-soluble drugs, however, are not readily eliminated until they are metabolized to more polar, less lipid-soluble compounds.

Renal Excretion. Excretion of drugs in the urine involves three processes: passive glomerular filtration, active tubular secretion and reabsorption, and passive tubular diffusion. The amount of drug entering the tubular lumen by *filtration* is dependent on the filtration rate and the degree of plasma protein binding. In the proximal renal tubule, strong organic acids and bases are added to the glomerular filtrate by active, carrier-mediated tubular *secretion*. Many organic acids, such as penicillin, are transported by the system that secretes naturally occurring substances such as uric acid; many organic bases, such as tetraethylammonium, are transported by another system that secretes choline, histamine, and other naturally occurring bases. Ionic forms of some weak electrolytes, such as salicylate, chlorothiazide, and quinine, are also secreted into the tubular lumen by these mechanisms. The characteristics of tubular transport systems are described in detail in Chapter 41.

In the proximal and distal tubules, the nonionized forms of weak acids and bases undergo reabsorption or excretion by *passive diffusion*. By definition, a passive diffusion mechanism is potentially bidirectional, and drugs may diffuse across tubular cells in either direction, depending upon the concentrations of the drug and the pH on the two sides of the tubular cells. Under normal conditions, even when the pH gradient in the distal tubule favors diffusion into the urine, the net effect is reabsorption, since the bulk of the drug will diffuse out of the urine as reabsorption of strong electrolyte and water creates a concentration gradient of the nonionized form in the direction of urine to blood. Passive reabsorption of the ionized form may also occur, but the proportion is considerably less than that of the nonionized form. When the tubular urine is more alkaline than plasma, weak acids are excreted more rapidly, primarily because of a decrease in net passive reabsorption. When the tubular urine is more acid than plasma, the excretion of weak acids is reduced. The effects of alkalinization and acidification of the urine on the excretion of weak bases are the opposite of those on the excretion of weak acids. In poisoning, the excretion of some drugs can be hastened by appropriate alkalinization or acidification of the urine. (*See*

Orloff and Berliner, 1961; Mudge and Weiner, 1963.)

Hepatic and Fecal Excretion. Many metabolites of drugs formed in the liver are excreted into the intestinal tract in the *bile*. The metabolite may be excreted in the feces, but the greater portion is usually reabsorbed into the blood and ultimately excreted in the urine. For example, diphenylhydantoin is metabolized in the liver, chiefly to hydroxydiphenylhydantoin; all of this metabolite enters the bile and hence into the intestinal tract, but it is subsequently reabsorbed and is ultimately excreted in the urine. The metabolite of colchicine, in contrast, is secreted in the bile and excreted only in feces, since absorption across the intestinal mucosa does not occur. Both strong organic acids and strong organic bases are actively transported from hepatic cells into bile by processes similar to those that transport these same substances across renal tubules.

Excretion by Other Routes. Excretion of drugs into *sweat* and *saliva* takes place by similar mechanisms, but both routes are quantitatively unimportant. Excretion is dependent mainly upon diffusion of the nonionized, lipid-soluble form of drugs through the epithelial cells of the glands, since the ionized forms pass only slowly into these secretions. Thus, pK_a of the drug and pH of the primary secretion formed in the acini of the glands are important determinants of secretion rate. Nonionic reabsorption of the drug from the primary secretion probably also occurs in the ducts of the glands, and this process is dependent on the pH of the fluid in the duct lumen. It is not known whether active secretion of drug occurs across the ducts of the gland. Lipid-insoluble compounds, such as urea and glycerol, appear to enter saliva and sweat at rates proportional to their molecular weight, presumably by filtration through aqueous channels in the membrane. Drugs excreted in the saliva enter the mouth, where they are usually swallowed. Their fate thereafter is the same as that of drugs taken orally.

The same principles apply to excretion of drugs in *milk*. Since milk is more acidic than plasma, basic compounds may be concentrated in this fluid. In contrast, the concentration of acidic compounds in milk is lower than in plasma. Nonelectrolytes, such as ethanol, urea, and antipyrine, readily enter milk and reach the same concentration as in plasma, independently of the pH of the milk. (*See* summary by Stowe and Plaa, 1968.)

MECHANISMS OF DRUG ACTION

The most fundamental aspect of pharmacodynamics is that which deals with the mechanisms of drug action. Before summarizing some general concepts pertaining to this fascinating subject, it is helpful to emphasize the distinction between drug action and drug effect, and clearly to define the objectives of analysis of drug action.

Although often considered as synonyms, the terms *action* and *effect* have useful pharmacological connotations that should be preserved. With certain obvious exceptions, most drugs are thought to produce their effects by combining with enzymes, cell membranes, or other specialized functional components of cells. Drug-cell interaction is presumed to alter the function of the cell component and thereby initiate the series of biochemical and physiological changes that are characteristic of the drug. Only the initial consequence of drug-cell combination is correctly termed the *action* of the drug; the remaining events are properly called drug *effects*. The objectives of analysis of drug action are identification of the primary action, delineation of the details of the chemical reaction between drug and cell; and characterization of the full action-effects sequence. Only the complete analysis provides a truly satisfactory basis for the therapeutic use of the drug, but this is an ideal that is infrequently attained.

Structure-Activity Relationship. The actions of a drug are intimately related to its chemical structure. The relationship is frequently quite specific, and relatively minor modifications in the drug molecule may result in major changes in pharmacological properties. Exploitation of structure-activity relationship has led to the synthesis of many valuable therapeutic agents. Since changes in molecular configuration need not alter all actions and effects of a drug equally, it is sometimes possible to develop a congener with a more favorable therapeutic index or more acceptable secondary characteristics than those of the parent drug. In addition, effective therapeutic agents have been fashioned by developing structurally related competitive antagonists of other drugs or of endogenous substances known to be important in biochemical or physiological function.

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ship appears quite broad. For example, many chemically dissimilar drugs exhibit local anesthetic activity, and compounds of totally different chemical structure produce similar CNS depressant effects. These examples do not negate the significance of the structure-activity relationship, but merely emphasize that much remains to be learned of the basic mechanisms of action of most drugs and that they may produce overtly similar effects by more than a single mechanism.

Drug Receptors. The cell component directly involved in the initial action of a drug is usually termed its *receptive substance* or, simply, its *receptor*. The chemical groups that participate in drug-receptor combination and the adjacent portions of the receptor that favor or hinder access of the drug to these active groups are known as *receptor groups* or *receptor sites*. The formation of the drug-receptor complex, the initial action of the drug, and the early events in the action-effects sequence are sometimes referred to collectively as the *receptor process*. Drug-cell interactions that do not initiate drug action, such as the binding of drugs to plasma and cell proteins and to enzymes concerned with biotransformation and transport of drugs, are said to involve *secondary receptors, silent receptors, sites of loss, storage sites*, or *drug acceptors*.

Receptors and Theories of Drug Action. As early as 1878, even before he coined the term *receptive substance*, Langley suggested that drug-cell combinations, and hence the actions and effects of drugs, were probably governed by the law of mass action. This view was extensively developed by A. J. Clark in the 1920s, and it remains the keystone of most theories of drug action. Thus, theories of drug action are quite similar to theories of enzyme action and are essentially identical when a drug serves as an antimetabolite or enzyme inhibitor.

A drug that combines with receptors and initiates an action-effects sequence is said to possess both *affinity* and *efficacy* (or *intrinsic activity*) and is termed an *agonist*; one that combines with the same receptors but fails to initiate drug action is considered to lack efficacy and acts as a *competitive antagonist*. An agonist that produces a smaller maximal effect than do other agonists that act on the same receptor is said to have intermediate efficacy and is termed a *partial agonist*. The effects of a partial agonist and those of a full agonist acting on the same receptor may be additive, or the partial agonist may antagonize the full agonist, depending upon their relative concentrations. Many examples of partial agonists have been noted among the autonomic agents, and nalorphine and related so-called opioid antagonists appear to be partial agonists for certain opioid receptors. Why various drug-receptor combinations differ in their ability to initiate drug action remains to be explained.

The major value of receptor theory is that of providing a conceptual framework for analysis of mechanism of drug action. However, it must be emphasized that drug action is defined not by an equation derived from receptor theory that relates dose and effect or that describes the pattern of drug interaction, but only by identification of the role of drug receptors in normal cellular function and by characterization of the action-effects sequence.

For certain applications of receptor theory, it is necessary to assume some relationship between drug-receptor interaction and intensity of drug effect. In the classical receptor theory developed by Clark, it was assumed that drug effect is proportional to the fraction of receptors occupied by drug, and that maximal effect results when all receptors are occupied. Neither of these assumptions is likely, and subsequent modifications of *occupation theory* have assumed other relationships between receptor occupation and drug effect and have permitted the possibility that maximal effect may be achieved when only a portion of receptors is occupied. The latter concept is described as that of *spare receptors* or *receptor reserve*. It has also been proposed that drug effect may be a function not of receptor occupation but of the rate of drug-receptor combination. This *rate theory* is attractive because it relates drug efficacy to the rate of dissociation of the drug-receptor complex and explains certain other aspects of the time course of drug effect.

Although receptors for most drugs have yet to be identified, there is little doubt that drug-cell combinations that obey mass-law kinetics are involved in drug actions. The many discrete relationships between chemical structure and biological activity and the competitive interaction of chemically similar drugs are difficult to explain except in these terms. Receptor groups, like the active centers of enzymes, are thought to be carboxyl, amino, sulfhydryl, phosphate, and similar reactive groups

spatially oriented in a pattern complementary to that of the drugs with which they react. The binding of drug to receptor is thought to be accomplished mainly by ionic and other relatively weak, reversible bonds. Occasionally, firm covalent bonds are involved, and the drug effect is only very slowly reversible.

Classification of Receptors and Drug Effects. The statement that a drug activates or blocks specified drug receptors should not be mistaken as a description of its mechanism of action. Drug receptors are classified on the basis of the effect, or lack of effect, of selective antagonists and by the relative potencies of representative agonists. For example, the effects of acetylcholine that mimic those of the alkaloid muscarine and that are selectively antagonized by atropine are termed *muscarinic effects.* Other effects of acetylcholine that mimic those of nicotine and that are not readily antagonized by atropine but are selectively blocked by other drugs are described as *nicotinic effects.* By *extension,* these two types of cholinergic effects are said to be mediated by muscarinic or nicotinic receptors. Such classification of receptors contributes little to delineation of mechanism of drug action. Nevertheless, since the effects and receptors in the various tissues have been classified, a statement that a drug activates or blocks a specified type of receptor is a succinct summary not only of its spectrum of effects but also of the drugs that it will antagonize, or that will antagonize it. The use of receptor terminology to describe drug effects has been developed most extensively in connection with neurotransmitters and autonomic drugs, but the same concepts apply to other classes of drugs. However, receptor terminology is useful only if the drugs chosen for classifying drug receptors are selected in a meaningful manner, and if the effects of these drugs have been well characterized.

Sites of Action. Some drugs act only on certain cells, tissues, or organs and thereby exert *localized* effects. Other drugs act on most cells of the body and thus exert *generalized* effects. Localization of drug effect does not necessarily depend upon selective distribution of the drug. Some drugs act *extracellularly;* others, at the *cell surface;* and still others, *intracellularly.*

Certain drugs act *directly* on effector cells; others influence effector cells *indirectly.* A substance may lower blood pressure directly by inhibition of vascular smooth muscle, or indirectly by acting on the vasomotor center or on autonomic ganglia. Drugs may also produce their effects indirectly by promoting or preventing the action of another substance. Many of the effects of neostigmine are due to the preservation of acetylcholine at neuroeffector junctions, as a result of inhibition of the enzyme that normally destroys this neurotransmitter; and many of the effects of atropine are the result of competition with acetylcholine for its effector sites.

Biochemical and Biophysical Mechanisms of Action. The clinical effects of a drug can usually be described in terms of alterations of physiological function, and they can often be correlated with biochemical and biophysical effects of the agent. However, the primary actions of drugs have been elucidated in relatively few cases. Not infrequently, analysis of drug action is limited by available physiological and biochemical knowledge. Elucidation of basic cellular function and further exploration of drug action then proceed in parallel, often with the drug serving as an indispensable tool.

There are a few instances in which the mechanism of drug action is evident. Magnesium sulfate acts as a cathartic because the magnesium and sulfate ions are poorly absorbed and, therefore, exert an osmotic force that retains water in the lumen of the bowel. Also, dimercaprol (BAL) and other agents are useful in therapy of heavy-metal intoxication because they chelate with these metals to render them nontoxic.

Drugs may act by influencing the bound forms of endogenous, physiologically active substances. For example, the pharmacological effects of some drugs have been traced to the displacement of hormones, such as thyroxine, estradiol, and cortisol, from their binding sites on plasma proteins. Similarly, the pharmacological effects of tyramine result from *release* of norepinephrine from granules in adrenergic nerve terminals, and some antiadrenergic agents prevent the release of stored mediator. Other antiadrenergic drugs inhibit binding or decrease synthesis of norepinephrine and *deplete* the neurotransmitter from the nerve endings.

Drugs may also act by influencing any of the steps involved in maintenance of normal cell function. Thus, they may enhance or prevent the entrance into the cell of substances necessary for

[Chap. 1]

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energy production, synthetic reactions, or maintenance of the osmotic and electrical properties of the cell. These changes may be brought about either by interactions with specific drug receptors or by changing the structure of the surrounding solvent without interacting with the cells directly; general anesthetics may act in this latter way. *Transport* of glucose into cells is enhanced by insulin, the uptake of choline by nerve cells is blocked by hemicholinium, active transport of sodium and potassium is inhibited by digitalis, and the *permeability* of the neuromuscular junction to ions is increased by acetylcholine. Many drugs inhibit or enhance *enzyme* activity or influence energy production. For example, epinephrine enhances liver phosphorylase activity by accelerating the formation of cyclic adenosine-3′,5′-monophosphate through the activation of the enzyme adenyl cyclase. Agents such as salicylates, thyroxine, and 2,4-dinitrophenol in high dosage uncouple phosphorylation from oxidation. Uncoupling by thyroxine has been attributed to a change in the spatial arrangement of mitochondrial enzymes as a result of swelling; but this is not the primary action of the hormone, since the mechanism whereby the swelling of mitochondria is produced must still be elucidated. In physiological doses, however, thyroxine causes increased synthesis of nuclear, ribosomal, and cytoplasmic soluble RNA and increases activity of nuclear RNA polymerase and other enzymes involved in nucleic acid and protein synthesis. Hormones, streptomycin and other antibiotics, and the purine and pyrimidine antimetabolites also appear to act by affecting template molecules involved in cell synthetic processes. Since there are a number of steps involved in these processes, many sites of drug action are possible. Thus, insulin is thought to increase the ability of ribosomes to bind and translate messenger RNA, possibly by modification of ribosomal structure, and aldosterone is thought to regulate active sodium transport by an as-yet-unexplained effect of DNA-directed RNA synthesis and *de-novo* synthesis of proteins.

CHARACTERIZATION OF DRUG EFFECTS

The effects of drugs are variously expressed as biochemical or physiological changes, or as the occurrence or relief of clinical symptoms. Thus, drugs are described as anticoagulants, vasoconstrictors, psychotomimetics, analgesics, and so forth. However, for evaluation and comparison of drugs, their effects must be related to dosage, and they must be further characterized in terms of maximum efficacy, variability, and selectivity. Equally important is delineation of the time course of drug effect, both after administration of single doses of the drug and during chronic medication.

Time Course of Drug Effect. The various effects of a drug need not have the same time course. Each effect is commonly characterized by its *latency, time of peak effect,* and *duration.* Although also modified indirectly by the factors that influence duration of effect, the latent period between administration of the drug and onset of effect is determined largely by the route of administration and the rates of absorption and distribution of the drug. The rate of biotransformation to an active metabolite may also be important. Similarly, although also modified by continuing absorption from the site of administration, duration of effect is determined primarily by the rates of inactivation and excretion of the drug. In addition, redistribution from its locus of action to storage sites, physiological reflexes, and development of drug tolerance may also contribute to termination of drug effect. In most cases, as dosage is increased, latency is reduced and duration is prolonged. Duration of effect is also conveniently expressed in terms of the *half-times* for decline of effect and, when it closely parallels drug effect, for decline of plasma concentration of the drug. These indices of duration of effect are particularly useful for describing dosage schedules for chronic medication.

The Dose-Effect Relationship. Ideally, the relationship between dose and effect is based upon the effects attained under equilibrium conditions. However, in practice, the dose-effect relationship is commonly derived from the peak effects after single doses of the drug. There is no single characteristic relationship between intensity of drug effect and drug dosage. A dose-effect curve may be linear, concave upward, concave downward, or sigmoid. Moreover, if the observed effect is the composite of several effects of the drug, such as the change in blood pressure produced by a combination of cardiac, vascular, and reflex effects, the dose-effect curve need not be monotonic. However, a composite dose-effect curve can usually be resolved into simple curves for each of its components; and simple dose-effect curves, whatever their precise shape, can be viewed as having four characterizing parameters: potency, slope, maximum efficacy, and vari-

ability. These are illustrated in Figure 1–3 for the common sigmoid log dose-effect curve. The logarithmic transformation of dosage is often employed for the dose-effect relationship, because it permits display of a wide range of doses on a single graph, and because it has certain mathematical advantages when dose-effect curves are compared.

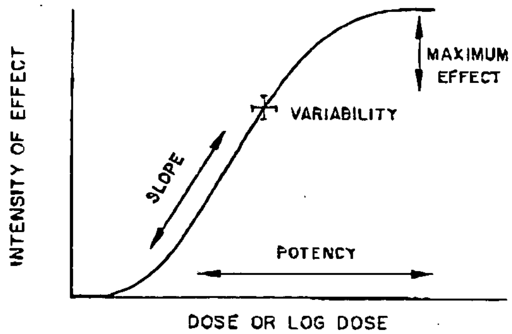

Figure 1–3. *The dose-effect relationship.*

Representative dose-effect curve, illustrating its four characterizing parameters (*see* text for explanation).

Potency. The location of its dose-effect curve along the dose axis is an expression of the potency of a drug. Potency is influenced by the absorption, distribution, biotransformation, and excretion of a drug, as well as being determined by its inherent ability to combine with its receptors. Potency is a relatively unimportant characteristic of a drug, since it makes little difference whether the effective dose of a drug is 1 μg or 100 mg, as long as the drug is administered in appropriate dosage. Potency is not necessarily correlated with any other characteristic of a drug, and there is no justification for the view that the more potent of two drugs is clinically superior. Low potency is a disadvantage only if the effective dose is so large that it is awkward to administer. Extremely potent drugs, particularly if they are volatile or are absorbed through the skin, may be hazardous and may require special handling.

For therapeutic applications, the potency of a drug is necessarily stated in *absolute* dosage units (25 μg, 10 mg/kg, etc.); for comparison of drugs, *relative potency,* the ratio of equieffective doses ($\frac{1}{10}$ ×, 5 ×, etc.) is a more convenient expression.

Slope. The slope of the more-or-less linear, central portion of the dose-effect curve is of more theoretical than practical importance. For example, a steep dose-effect curve for a CNS depressant implies that there is a small ratio between the dose that produces coma and that which causes mild sedation, and that excessive or inadequate effect may occur if the dose of the drug is not carefully adjusted. Nevertheless, many factors influence the margin of safety of a drug and the variability of its effects, and these characteristics of a drug are properly expresed by methods that summarize the contributions of all factors (*see* below). It should be noted that doubling the dose of a drug does not necessarily produce twice the effect, and the quantitative relationships between dose and effect should not be confused in this manner.

The significance of the slope and shape of the dose-effect curve in analysis of drug action is beyond the scope of the present discussion. However, it deserves passing mention that mere parallelism of their dose-effect curves is not a reliable basis for concluding that different drugs produce their effects by the same mechanism.

Maximum Efficacy. The maximum effect produced by a drug, even at very large dosage, is termed its *ceiling effect* and is referred to as its maximum efficacy or, simply, efficacy. Maximum efficacy of a drug may be determined by its inherent properties and be reflected as a plateau in the dose-effect curve, but it may also be imposed by other factors. If the undesired effects of a drug limit its dosage, its efficacy will be correspondingly limited, even though it is inherently capable of producing a greater effect. Maximum efficacy of a drug is clearly one of its major characteristics. One of many important differences between morphine and aspirin is that the opiate has sufficient efficacy to provide relief of pain of nearly all intensities, whereas the salicylate is effective only against mild-to-moderate pain.

Efficacy and potency of a drug are not necessarily correlated, and these two characteristics of a drug should not be confused.

Biological Variation. The more important factors that modify drug effect are summarized later in this chapter. However, even when all known sources of variation are controlled or taken into account, drug effects are

never identical in all patients, or even in a given patient on different occasions. A dose-effect curve applies only to a single individual under carefully controlled conditions or to the average individual. The perpendicular brackets in Figure 1–3 indicate that biological variation of the dose-effect relationship can be visualized in either of two ways. The vertical bracket expresses the fact that a range of effects will be produced if a given dose of a drug is administered to a group of individuals; alternatively, the horizontal bracket expresses the fact that a range of doses is required to produce a specified intensity of effect in all individuals.

Dose-Percent Curve. The dose of a drug required to produce a specified intensity of effect in an individual is termed the *individual effective dose.* Individual effective doses of most drugs are lognormally distributed, which means that the familiar *normal curve* of variation is obtained if the logarithms of the individual effective doses for a group of patients are expressed as a frequency distribution. A cumulative frequency distribution of individual effective doses, that is, the percentage of individuals that exhibit the effect plotted as a function of logarithm of dose, is known as a dose-percent curve. Although also a sigmoid curve, the dose-percent curve is not a dose-effect curve but merely an expression of individual variability for a single effect.

The dose of a drug required to produce a specified intensity of effect in 50% of individuals is known as the *median effective dose* and is abbreviated ED50 (*not* ED_{50}). If death is the end point, the median effective dose is termed the *median lethal dose* (LD50). The doses required to produce the stated effect in other percentages of the population are similarly expressed (ED20, LD90, etc.). Similar notations are also often used to refer to the dose of a drug required to produce a stated fraction of the maximum effect or a stated intensity of effect. These conflicting uses of the same abbreviations should not be confused.

Terminology. Specific terms are used to refer to individuals who are unusually sensitive or unusually resistant to a drug, and to describe those in whom a drug produces an unusual effect. If a drug produces its usual effect at unexpectedly low dosage, the individual is said to be *hyperreactive.* Hyperreactivity should not be described as *hypersensitivity,* since this term is usually used to refer to the pattern of effects associated with drug allergy. Hyperreactivity to a drug should be termed *supersensitivity* only if the increased sensitivity is the result of denervation. If a drug produces its usual effect only at unusually large dosage, the individual is said to be *hyporeactive.* Hyporeactivity is also described as *tolerance,* but this term has the connotation of hyporeactivity acquired as the result of prior exposure to the drug. Tolerance that develops rapidly after administration of only a few doses of a drug is termed *tachyphylaxis.* Hyporeactivity should be described as *immunity* only if the acquired tolerance is the result of antibody formation.

An *unusual effect* of a drug, of whatever intensity and irrespective of dosage, that occurs in only a small percentage of individuals is often termed *idiosyncrasy.* However, this term is frequently considered a synonym for drug allergy and has so many other connotations that it probably should be abandoned. Unusual effects of drugs have also been called *meta reactions,* but this term has not gained wide acceptance. Perhaps unusual effects of drugs are best described as such or by terms that refer to the underlying mechanism; they are often types of drug allergy or a consequence of genetic differences.

Selectivity. A drug is usually described by its most prominent effect or by the action thought to be the basis of this effect. However, such descriptions should not obscure the fact that *no drug produces only a single effect.* Morphine is correctly described as an analgesic, but it also suppresses cough and causes sedation, respiratory depression, constipation, bronchiolar constriction, release of histamine, antidiuresis, and a variety of other effects. A drug is adequately characterized only in terms of its full *spectrum of effects.* The relationship between the desired and undesired effects of a drug is termed its *therapeutic index, margin of safety,* or *selectivity.* Rarely is a drug sufficiently selective to be described as being *specific.* For therapeutic applications, selectivity of a drug is clearly one of its more important characteristics.

Because the ideal drug produces its desired effect in all patients without causing toxic effects in any, and because dose-percent curves need not be parallel, it is sometimes argued that therapeutic index should be defined as the ratio between the minimum toxic dose and the maximum effective dose. However, minimum and maximum effective doses cannot be estimated with precision, and therapeutic index is usually defined as the ratio between the median toxic dose and the median effective dose (TD50/ED50). In laboratory studies, therapeutic index is often based upon the median lethal dose (LD50/ED50). In clinical studies, drug selectivity is often expressed indirectly by summarizing the pattern and incidence of adverse effects produced by therapeutic doses of the drug and by indicating the proportion of patients who were forced to decrease drug dosage or discontinue medication because of adverse effects. These indirect procedures are often adequate, but comparison of dose-effect curves for desired and undesired effects is more consistently meaningful and is preferred whenever feasible.

A drug does not have a single therapeutic index, but many. The margin of safety of aspirin for relief of headache is greater than its margin of safety for relief of arthritic pain, since the latter use requires larger dosage. Similarly, several therapeutic indices can be calculated for each desired effect. A synthetic opiate substitute may cause less constipation than morphine and yet afford no advantage over the parent compound with regard to respiratory depression or sedation. Moreover, a drug may be selective within one context yet still be nonselective within another. The antihistamines are correctly described as selective antagonists of histamine, yet none of these drugs produces this selective peripheral effect without also causing significant central sedation. Finally, a drug may be correctly described as having an adequate margin of safety in most patients, but this description is meaningless for the patient who exhibits an unusual response to the drug. Penicillin is essentially nontoxic in the majority of patients, yet it has caused death in those who have become allergic to it.

Biological Assay. Alkaloids and other highly purified drugs obtained from plants and animals can usually be standardized by their chemical or physical properties. However, drugs that are only partially purified, such as certain digitalis preparations and various hormones, vitamins, antibiotics, and vaccines, must be standardized by their biological properties. The estimation of the relative potencies of such preparations by comparison of their biochemical, pharmacological, or toxic effects is termed *biological assay* or, simply, *bioassay*. An effect of the preparation is compared with that of a *reference standard* under carefully controlled conditions. The reference standard is usually a highly purified, often crystalline, preparation of the substance being assayed. The potency of a preparation standardized by biological assay is usually expressed in units, or weight equivalent, of the reference standard. Official drugs have legally required methods of assay, and both the assay procedure and the reference standard are rigidly defined. These are often the same as those specified by an international committee. Nonofficial preparations that are assayed by different methods or against different reference standards may vary considerably in relative potency.

FACTORS THAT MODIFY DRUG EFFECTS AND DRUG DOSAGE

Many factors modify the effects of drugs. Some of these, such as the development of drug allergy, result in *qualitative* differences in the effects of a drug and may preclude its safe use. Others produce only *quantitative* changes in the usual effects of the drug and can be offset by appropriate adjustment of dosage. These variables must be taken into account before a drug is prescribed, and a stated *therapeutic dose* of a drug must be viewed only as the anticipated dose for the average patient or that from which to estimate the dose for an individual patient. Indeed, the optimal dosage for many drugs is determined for each patient only by careful monitoring of drug effect. It is for this reason that it is appropriate to state that *the dose of a drug is "enough."* The physician's legal responsibility if he deviates from dosages recommended in official compendia or in the manufacturer's package insert has been summarized in a Council Conference (1969).

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The following are the more important factors that influence the therapeutic dose of a drug.

Body Weight. The ratio between the amount of drug administered and body weight influences the concentrations of the drug at its sites of action. Therefore, drug dosage should be suitably adjusted, particularly for abnormally lean or obese individuals. For adjustment of drug dosage over a wide range of body sizes, body surface area is often a more satisfactory index than is body weight.

Age. Children are often more sensitive than adults to drug-induced changes in water and electrolyte or acid-base metabolism. Special precaution must also be taken in the use of hormones or other drugs that might influence growth and development. Very young infants, particularly premature babies, may be abnormally sensitive to many drugs because of immaturity of renal function or of the enzymatic mechanisms for drug inactivation, or because of incomplete development of the blood-brain barrier. The effects of drugs on the immature brain may also be somewhat unusual.

Although drug dosage for older children may be calculated as a fraction of the adult dose on the basis of body weight or estimated body surface area, that for younger children, and especially for infants, should be learned as such and not calculated by formula. Unfortunately, optimal doses for children have been established for relatively few drugs.

Elderly individuals may respond to drugs in a somewhat abnormal manner, often because of impaired ability to inactivate or excrete drugs, or because of other concurrent pathology.

Sex. Women are thought to be more susceptible than men to the effects of certain drugs, in part because of smaller size. In some instances, this difference is considered sufficient to necessitate reduction in dosage. In addition, the subjective effects of drugs may be somewhat different in the two sexes. During pregnancy, caution is necessary in the administration of drugs that might affect the uterus or fetus. A wise precaution is to avoid the use of all drugs except those essential to maintain pregnancy. Activity of the hepatic microsomal enzymes involved in drug biotransformation is only slightly influenced by sex.

Route of Administration. Inasmuch as rate and extent of absorption and sometimes pattern of biotransformation differ with the route of administration, dosage must be adjusted to take this factor into account. The intravenous dose of a drug is often smaller than the subcutaneous, and this, in turn, is usually smaller than the oral dose.

Time of Administration. The time at which a drug is administered sometimes influences dosage. This is especially true for oral therapy in relation to meals. Absorption proceeds more rapidly if the stomach and upper portion of the intestinal tract are free of food, and an amount of drug that is active before a meal may be ineffective if given after eating. On the other hand, irritating drugs are better tolerated if food is in the stomach. Diurnal and seasonal variations in the effects of drugs are well recognized in animals and may also be important in man.

Rate of Inactivation and Excretion. If function of the organs concerned in inactivation or excretion of a drug is impaired, both intensity and duration of effect of a given dose of a drug may be greater than desired, and toxic effects may occur. Thus, it is of practical importance to know the mechanisms by which a drug is inactivated or eliminated, and to make appropriate adjustment in drug dosage for patients in whom these mechanisms are impaired.

Tolerance. Tolerance may be acquired to the effects of many drugs, especially the opioids, barbiturates and other CNS depressants, nitrites, xanthines, and certain CNS stimulants. When this occurs, *cross-tolerance* may develop to the effects of pharmacologically related drugs, particularly those acting at the same receptor site, and drug dosage must be increased to maintain a given therapeutic effect. Since tolerance does not usually develop equally to all effects of a drug, the therapeutic index often decreases. However, there are also examples of the development of tolerance to the undesired effects of a drug and a resultant increase in its therapeutic index. After tolerance develops, normal sensitivity may be regained only by suspending drug administration. For most drugs, the development of tolerance can be minimized by initiating therapy with the lowest effective dose and by avoiding continuous administration of the drug at regular intervals. In contrast, the emergence of resistant microorganisms during chemotherapy is favored when only minimally effective dosage or intermittent medication is employed.

The mechanisms involved in the development of tolerance are only partially understood. In animals, tolerance often occurs as the result of induced synthesis of the hepatic microsomal enzymes concerned in drug biotransformation; the possible significance of this *drug-disposition tolerance* during chronic medication in man is an area of active investigation. The most important factor in the development of tolerance to the opioids, barbiturates, and ethanol is some type of neuronal adaptation vaguely referred to as *cellular* or *pharmacodynamic tolerance*. Tachyphylaxis, such as that to histamine-releasing agents and to the sympathomimetic amines that act indirectly by releasing norepinephrine, has been attributed to depletion of available mediator, but other mechanisms have also been proposed.

Physiological Variables. Changes in water and electrolyte balance, acid-base status, body temperature, and other physiological factors may also modify the effects of drugs. Unfortunately, no simple summary of the effect of these variables is possible. Metabolic acidosis reduces the saluretic effects of

the carbonic anhydrase inhibitors but increases those of the organic mercurials. Similarly, hypothermia decreases blockade of neuromuscular transmission by tubocurarine but increases that by succinylcholine. Likewise, the effects of drugs may be increased or decreased after denervation.

The effects of drugs, just as those of disease, may be manifest as encroachment upon *physiological reserve* rather than as an overt effect, and this factor must also be considered when drugs are prescribed. Ganglionic blocking agents and other drugs that impair compensatory sympathetic reflexes may have minimal effects upon the blood pressure of a recumbent individual, yet cause orthostatic collapse when he assumes an upright posture. This principle is important in establishing proper dosage of drugs in the therapy of hypertension and in selecting drugs for preanesthetic medication. Similarly, respiratory depression may be manifest primarily as respiratory acidosis with only minimal reduction of rate or depth of breathing or of alveolar ventilation. Failure to appreciate this fact is often responsible for underestimation of the depressant effects of drugs on respiration.

Pathological State. The effects of certain drugs are also considerably modified by pathological conditions. Patients with chronic pulmonary disease or increased intracranial pressure are often unusually sensitive to morphine and other respiratory depressants. Conversely, the hyperthyroid individual can tolerate larger doses of morphine than can the normal person, but he is responsive to a dose of epinephrine that would scarcely affect a person with normal thyroidal function. *Nutritional* deficiencies may also modify the effects of drugs.

Activity of the hepatic microsomal enzymes involved in drug metabolism may be reduced in certain types of hepatic disease. Unfortunately, reduced microsomal activity is not consistently correlated with impaired hepatic function detected by the conventional functional tests.

Milieu. Many drug effects, particularly those on mood and behavior, and the subjective effects of drugs in general, are often susceptible to environmental factors or the "set" imparted at the time of drug administration. The CNS depressants may improve psychomotor performance under one set of experimental or clinical conditions yet impair such performance under other conditions. Similarly, the subjective effects associated with dummy medication in controlled clinical trials vary widely, depending upon the effects of active medication being evaluated concurrently, the manner in which the subjective effects are elicited, and many other factors.

Genetic Factors. Genetic factors contribute to the normal variability of drug effects and are responsible for a number of striking quantitative and qualitative modifications of pharmacological activity. Many of these differences, such as the prolonged apnea in some patients after administration of usual doses of succinylcholine (a neuromuscular blocking agent) have been traced to genetic influences on the concentrations of enzymes involved in *drug biotransformation*. Other variations in drug effect, such as the greater incidence of drug-induced hemolytic anemia in non-Caucasians than in Caucasians, also have been found to be related to genetically determined enzyme patterns that modify the *actions of drugs*.

The objectives of *pharmacogenetics* include not only identification of differences in drug effects that have a genetic basis but also development of simple methods by which susceptible individuals can be recognized *before* the drug is administered.

Drug Interaction. The effects of a drug may be modified by prior or concurrent administration of another drug, and improved therapy is sometimes possible by judicious use of concurrent medication. However, serious adverse effects may also result from drug interaction. Since a patient frequently receives five or more drugs during the course of an illness, the possibility of unplanned combination of drug effects must also be considered. Drug interactions may arise either from alteration of the absorption, distribution, biotransformation, or excretion of one drug by the other, or from combination of their actions or effects.

Multiple-drug therapy is justified if it provides greater efficacy than can be achieved with full doses of single drugs, greater margin of safety, or more satisfactory onset or duration of effect. Sometimes a drug is administered in combination with another to antagonize an untoward effect. However, it is usually more desirable to reduce the dose of the toxic drug or to change medication, rather than to resort to combined medication. Whatever the rationale for multiple-drug therapy, the efficacy and safety of the combined medication must be evaluated in the same manner as for single drugs.

Drug Mixtures. A distinction must be made between the concurrent, but separate, administration of drugs and their administration together as a fixed-dose mixture. Occasionally, as illustrated by the sulfonamide mixtures, the use of fixed-dose mixtures is justified. However, most mixtures of drugs have distinct disadvantages, and their use may involve frank risk. Careful adjustment of dosage is almost always required to attain the maximum benefits from combined medication with a minimum of untoward effects. In some cases, proper timing of drug administration may also be important. The flexibility of dosage and timing essential for the success of combined medication is sacrificed if fixed-dose mixtures are used. In addition, the use of mixtures often complicates therapy, since, if toxic effects occur, it may be impossible to identify the component responsible, and all medication must then be discontinued. The use of mixtures also fosters multiple-drug therapy without first establishing the need for more than one drug. When this occurs, needless expense is imposed upon the patient, and he is unnecessarily exposed to the risk of toxicity from the superfluous components. Additionally, the indiscriminate use of drug mix-

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Terminology. Descriptions of the combined effects of drugs are often ambiguous, because the terms *addition, summation, synergism*, and *antagonism* are not employed consistently. The usage outlined here is that of Loewe (1953).

Two drugs are said to be *heterergic* for a particular effect if the effect is manifested by one of the drugs but is absent from the spectrum of the other. If the combined effects of heterergic drugs are greater than those of the active component alone, they are said to exhibit *synergism;* if the combined effects are less than those of the active component alone, the interaction is termed *antagonism*. Although often employed as a synonym for synergism, the term *potentiation* should be abandoned, since it has erroneously acquired the connotation of clinical superiority.

Heterergic synergism and antagonism often result from alterations in the distribution, biotransformation, or excretion of the active component. Heterergic antagonism may also involve interaction of the two drugs at the same receptor. If the antagonist acts reversibly, *competitive* antagonism results; if it acts irreversibly, the interaction is termed *nonequilibrium* antagonism. Receptor antagonism is usually *selective* for a given agonist. If the two drugs form an inactive complex, the interaction is termed *chemical* antagonism.

The interaction of drugs that have overtly opposite effects is termed *physiological* or *functional* antagonism; such antagonism is usually *nonselective* and *noncompetitive*.

If two drugs produce the same overt effect, they are termed *homergic*. Descriptions of the combined effects of such drugs are intended to indicate whether they are equal to, or greater or less than, those expected by simple addition. However, two types of additive behavior can be distinguished. If the drugs are close congeners that act on the same receptors (*e.g.*, epinephrine and norepinephrine), *doses* of one drug should substitute for those of the other, in proportion to their relative potency, over a wide range of combinations. Only drugs that exhibit this *dose addition* are properly described as *additive*. Deviations from dose addition are termed *supra-additive* or *infra-additive;* these latter descriptions imply that the drugs act by different mechanisms. If the combined *effects* of homergic drugs are equal to the sum of their individual effects, they are said to exhibit *effect addition* or *summation*. Deviations from summation are usually described as such. Drugs that act by the same mechanism and are additive in all combinations will exhibit summation only in certain combinations. The terms *synergism* and *antagonism* are best avoided for homergic drugs. Whatever terms happen to be used, most descriptions of the combined effects of drugs, particularly in the clinic, refer to effect addition rather than dose addition.

Dosage Schedules for Chronic Medication. To maintain the desired effect without cumulative toxicity during chronic medication, a *maintenance dose* of the drug must be administered at *dosage intervals* that balance the rates of inactivation and excretion. To avoid excessive fluctuation of effect between doses when the drug is rapidly absorbed, maintenance doses must be administered at frequent intervals, perhaps one sixth or less of the half-time for inactivation and excretion of the drug. Alternatively, the drug may be administered by constant intravenous infusion or as a repository preparation that provides slow, continuous absorption over an extended interval. If medication is initiated on a dosage schedule subsequently suitable for maintenance of effect, attainment of the desired effect is necessarily delayed. However, the effect may be achieved more promptly by administration of one or more *priming doses* somewhat larger than the subsequent maintenance doses. Chronic medication is often complicated by the development of drug tolerance and by the fact that medication is frequently interrupted during the hours of sleep. In addition, patients often fail to take medication as consistently and as regularly as directed.

DRUG TOXICITY

No drug is free of toxic effects. They may be trivial, but on occasion they are serious and may be fatal. Some appear promptly, but others may not develop until after prolonged medication. Still others occur only in certain patients or only in combination with other drugs. Some toxic effects of certain drugs, such as hemorrhage during anticoagulant medication, are an extension of the desired effects and can be avoided by proper adjustment of dosage. On the other hand, the desired and undesired effects of a drug may be different manifestations of the same primary action and thus be inseparable. In many instances, an effect of a drug that is sought in one patient becomes an undesired effect in another when the drug is employed for a different purpose. The incidence of untoward effects of drugs varies greatly and unfortunately cannot always be anticipated from animal studies.

Clinicians have long been aware of the drug-induced diseases. However, with the introduction into therapeutic practice of drugs of greater and broader efficacy, the problem of drug toxicity has increased, and it is now considered the most critical aspect of modern therapeutics. Not only is a greater variety of serious toxicity being uncovered, but also the average incidence of adverse effects of medication is increasing and unex-

pected toxic effects are occurring relatively frequently. There is an urgent need for the development of methods in animals that accurately predict the potential harmful effects of drugs in man. There is also need for better procedures for prompt collection, assessment, and dissemination of reports of clinical toxicity. However, adverse effects do not arise solely because of the inherent toxicity of drugs and the limitations of the methods for early detection of this toxicity. Many of the adverse effects could be avoided if drugs were used more carefully and more wisely. The physician should avoid a toxic drug if a less toxic one will suffice; and he should, if possible, avoid the use of concurrent medication and especially the use of drug mixtures, since one drug may affect the toxicity of another. Moreover, he must be aware of the potential hazards of the drugs that he uses, and he must be prepared to act promptly if toxicity occurs. He must be especially alert for the unexpected.

A brief consideration of several of the more serious drug-induced diseases will introduce the reader to this important aspect of pharmacology.

Drug Allergy (Hypersensitivity). Although the incidence of allergic reactions to most drugs is low, the list of known offenders is growing steadily, and drug allergy represents a major problem in the use of some drugs. In addition, there is an urgent need for reliable, safe methods for detecting susceptible individuals before drug administration. The development of such tests is complicated by the fact that metabolites of a drug, perhaps even minor metabolites that are not detected in usual biotransformation studies, as well as the drug itself and even trace impurities may serve as haptens.

Drug allergy may take many forms, including the full spectrum of immediate and delayed types of allergic reactions produced by foreign macromolecules. Skin reactions extend from mild rash to severe exfoliative dermatitis. Those of blood vessels range from acute urticaria and angioedema to severe arteritis with localized medial degeneration. Drug fever is an allergic phenomenon that very closely resembles serum sickness; it is manifested by fever, leukocytosis, arthralgia, and dermatitides. Rhinitis, asthma, and even anaphylactic shock are other familiar allergic responses that can be precipitated by drugs.

Hepatocellular damage, cholestatic jaundice, renal tubular necrosis, depression of hematopoietic functions, photosensitivity, and a number of additional adverse effects of drugs may also be manifestations of drug allergy.

Blood Dyscrasias. Leukopenia, granulocytopenia, aplastic anemia, hemolytic anemia, thrombocytopenia, and, in some cases, defects in the clotting factors are serious, sometimes fatal, complications of drug therapy. Although drug allergy is responsible for many of the cytopenias, certain of the blood dyscrasias are believed to result from a direct toxic effect of drugs on bone marrow. The most common basis for drug-induced hemolytic anemia is a genetically determined deficiency in red-cell glucose-6-phosphate dehydrogenase activity.

Hepatotoxicity and Nephrotoxicity. Because drugs are concentrated in the liver and kidney, damage to these organs by a direct toxic effect of drugs is not uncommon. Hepatotoxicity and nephrotoxicity may also occur as forms of drug allergy. Well-recognized adverse effects on the liver include hepatocellular toxicity, the potentially fatal viral hepatitis–like syndrome produced by the halogenated hydrocarbons and other drugs, and intrahepatic cholestasis, a type of hepatotoxicity that resembles obstructive jaundice and is produced by the phenothiazines, certain steroids, and a number of other agents. In addition, a variety of drugs precipitate hepatic coma in patients with liver disease. Glomerular-tubular damage is an important toxic effect of a number of drugs, including several antibiotics, and intrarenal precipitation of the less soluble sulfonamides is the major cause of the nephrotoxicity of these agents.

Teratogenic Effects. Although the thalidomide tragedy dramatically emphasized that drugs may adversely influence fetal development, there is still little reliable clinical information about the possible teratogenic hazard of most drugs. For this reason, all un-

necessary medication should be avoided during pregnancy. Moreover, since pregnancy is often not diagnosed at the time of greatest vulnerability of the fetus, all drugs not known to be reasonably safe on the basis of long usage should be avoided by women of childbearing age.

Behavioral Toxicity. This term refers to suppression of normal anxiety, reduction in motivation, impairment of memory and learning, distortion of judgment, nonpurposive or inappropriate behavior, and other adverse effects of drugs on mood, behavior, and psychological and psychometric functioning. Motor incoordination and impairment of ability to operate machinery or to drive a motor vehicle may also be considered a form of behavioral toxicity. The term has gained widest use in connection with psychopharmacological agents, but it applies to other drugs as well.

Drug Dependence and Drug Addiction. Any drug that alters mood or behavior is likely to be abused and is potentially capable of producing *drug dependence* upon repeated administration. The drugs that are commonly abused include the opioids; the barbiturates, other sedative-hypnotics, and ethanol; as well as the amphetamines, cocaine, and various other psychopharmacological agents. The characteristics of drug dependence vary with the agent involved. However, one characteristic common to all types of drug dependence is *psychic dependence*, a drive or craving that requires periodic or chronic administration of the drug for pleasure or for relief of discomfort. Another feature of some types of drug dependence is *physical dependence*, a state characterized by the appearance of physical symptoms when administration of the drug is supended. These symptoms are termed the *withdrawal* or *abstinence syndrome*. *Tolerance* is a characteristic of only certain types of drug dependence.

The term *drug dependence* was coined specifically to permit consideration of the pharmacological and medical aspects of *drug abuse* in isolation from the broader socioeconomic, moral, and legal aspects of the problem that are embraced by the term *drug addiction*. Unfortunately, the two terms continue to be used interchangeably. Abuse of drugs that adversely influence mood and behavior is a problem of increasing medical and social importance. The subject is considered in detail in Chapter 16.

Drug Poisoning. Accidental poisoning is a health problem of major significance. Several thousand deaths from chemical poisoning occur annually in the United States, and it is estimated that the number of nonfatal poisonings exceeds 1 million a year. More than one fourth of these fatalities and about one half of all poisonings occur in children under 5 years of age. The tragedy of this high incidence of poisonings in childhood is that most of them could be avoided.

The physician should assume an active role in *prevention* of poisoning. Parents cannot be too strongly urged to keep drugs out of reach of children, preferably in a "locked" cabinet, and to teach them that medicinals are not candy. The many common household articles that are poisonous should be made unavailable to children, and poisonous pesticides and herbicides should not be placed in the home. Indoctrination against accidental poisoning must also be directed to adults. They should be urged to read and heed labels on medicines, and they should be encouraged to discard residual prescription drugs that are no longer needed. Accidental poisoning among adults is not uncommon and usually results either from attempts at self-medication or from mistaking one drug for another.

The number and variety of drugs and chemicals that might be encountered in poisonings are enormous. However, the physician should become familiar with the details of treatment for the more commonly encountered household poisons. He should also be prepared to treat intoxication caused by agents frequently used for suicidal purposes, such as the barbiturates, other CNS depressants, and carbon monoxide, and to detect and treat those types of industrial intoxications that might occur in his community. Additionally, he should take advantage of the services and information available to him through his regional Poison Control Center.

Diagnosis of Drug Poisoning. The diagnosis of poisoning may be difficult, since there is scarcely a syndrome produced by a toxic agent that cannot simulate disease. However, there are a number of helpful signs and symptoms that assist in the detection of acute poisoning, particularly if this possibility is included in the differential diagnosis. In this connection, contaminated foods are often the etiological agent. The onset of symptoms is usually sudden and follows the taking of food, drink, or medicine by an individual who has been previously well. In some cases, several individuals may suffer from similar symptoms after partaking of the same food. In addition, many drugs leave a telltale odor, produce irritation of the mucous membranes of the mouth and throat, or cause typical pharmacological effects. As a final check, there are often simple chemical tests that can be performed to confirm the diagnosis.

The diagnosis of chronic poisoning is often more difficult. At times, the symptoms and signs are not sufficiently characteristic to point to the toxic agent, and laboratory procedures may be essential. In many cases, only painstaking probing of the patient's history, habits, daily activities, and working conditions leads to the diagnosis. The more uncommon types of industrial poisoning may go undiagnosed until irreparable injury has been suffered by the patient.

Treatment of Drug Poisoning. Most emergency therapy of drug poisoning is symptomatic, since success often depends upon celerity, and valuable time cannot be wasted in attempts at a positive identification of the specific cause of the patient's illness. However, therapy is facilitated if the responsible agent and the degree of exposure or the amount ingested can be determined. The usual steps taken are (1) supportive or symptomatic therapy, (2) termination of exposure and removal of the poison from the body, and (3) administration of antidotes.

√ Adequate *supportive therapy* is the most important aspect of the treatment of drug poisoning. Serial measurement and charting of the vital signs and important reflexes are helpful procedures by which to judge the progress of the intoxication, the response to therapy, and the need for additional treatment. The patient should be kept in bed and warm, under competent surveillance, and particular attention should be paid to the *respiration, circulation, hepatic and renal function,* and *acid-base and fluid-and-electrolyte balance.* This usually necessitates hospitalization.

Poisons that have been applied externally are best removed by copious *washing* with water, or with a suitable organic solvent if the poison is not water soluble. Induced vomiting, gastric lavage, and oral administration of *activated charcoal* may be employed to reduce further absorption of an ingested poison. The relative merits of these procedures have been reviewed by Gosselin and Smith (1966). Vomiting and gastric lavage are contraindicated in the treatment of poisoning by corrosive agents, strychnine, other convulsants, kerosene, or other hydrocarbon solvents.

Much emphasis has been placed on the use of *emetics,* since vomiting may be as productive as gastric lavage and can be instituted more promptly. However, such drugs are contraindicated in the unconscious patient, and they may not be effective if the patient has ingested a CNS depressant. *Ipecac syrup* is orally effective, but vomiting may not occur for 20 to 30 minutes. It is not dependable if the patient has previously received activated charcoal. *Apomorphine* acts more promptly and certainly, but it must be administered parenterally. It may also cause protracted emesis and CNS depression. Powdered mustard, administered orally as a suspension in warm water, is a household article that can be administered in an emergency. Mechanical stimulation of the oropharynx is unreliable and relatively ineffective.

Gastric lavage, when performed by a trained person, is an effective method for evacuation of the stomach. However, the procedure is time consuming and may not be reliable if the poison is insoluble. Care must be taken to prevent aspiration of gastric contents or lavage fluid into the lungs. After the stomach has been thoroughly emptied, a saline cathartic is sometimes administered through the stomach tube to diminish further intestinal absorption of the poison.

Activated charcoal, administered orally as a fine powder suspended in water, adsorbs a wide variety of chemicals (but not cyanide) and is an effective procedure for retarding absorption of ingested poisons. Since the combination of drug and charcoal is usually reversible, the gastric contents should be removed by lavage or by apomorphine-induced vomiting. The so-called universal antidote, a mixture of activated charcoal, magnesium oxide, and tannic acid, should be abandoned, since it is less effective than activated charcoal alone. Household articles that are somewhat useful for dilution or adsorption of poisons are milk, beaten egg white, flour and starch. Burnt toast is ineffective.

In some instances, the renal *excretion* of a drug can be increased by administration of an osmotic diuretic and by appropriate acidification or alkalinization of the urine. Volatile substances that are excreted by the lungs can often be more rapidly eliminated by stimulating respiration, and the inhalation of carbon dioxide is sometimes employed for this purpose. In cases of severe drug intoxication, elimination of the drug by *peritoneal dialysis* or by use of the *artificial kidney* is a highly effective procedure.

Antidotes serve only a limited role in the treatment of drug poisoning, even when the poison has been identified, because there are safe, effective, selective antagonists for relatively few drugs. Those that are available for individual poisons and drugs are discussed throughout the text. Outstanding examples are the use of chelating agents in metal poisoning, nitrites and thiosulfate in cyanide intoxication, methylene blue in methemoglobinemia, and atropine and reactivators of acetylcholinesterase in poisoning by insecticides and other drugs that inhibit the enzyme.

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DEVELOPMENT, EVALUATION, AND CONTROL OF DRUGS

The average practitioner is not usually directly concerned in the evaluation of drugs. Nevertheless, he must know something about the development and evaluation of therapeutic agents, since this knowledge has an important bearing on his attitude toward new drugs and their use. The physician must also be familiar with the laws that regulate the use of medicinals.

Sources and Discovery of New Drugs. The earliest medicinals were crude powders, juices, or extracts from animal, plant, and mineral sources, and these continued to be the only drugs until early in the nineteenth century. However, once advances in chemistry permitted the isolation, purification, and identification of the active constituents of these substances, it was just a step to structural modifications of existing drugs, the synthesis of new ones, and the beginning of the modern age of pharmacotherapy.

New therapeutic agents are discovered by screening, structural modification of established drugs, or accident. Screening refers to the testing of random compounds for selected types of pharmacological activity. The testing of thousands of soil samples for antibiotic activity is an example of this time-consuming, somewhat unsophisticated, relatively inefficient approach. However, it has the important advantage that it can uncover valuable new chemical classes of drugs.

Structural modifications of an established drug often yield congeners that differ only insignificantly from the parent and are aptly termed "me-too" drugs, the marketing of which is hard to justify. However, as discussed in the section on structure-activity relationship above, such alterations do often yield a congener with pharmacological properties significantly different from those of the parent compound. For example, the first three sulfonamides introduced as chemotherapeutic agents, a milestone in the history of drug therapy, are now obsolete. Another particularly fruitful approach has been the structural modification of endogenous molecules involved in cellular metabolism. This has led to the discovery of useful antimetabolites. Rapid advances in the elucidation of basic cellular function make it likely that more and more drugs of this type will be developed in the future.

Many significant advances in therapy in the past have resulted from fortuitous discovery of new drugs or new uses of established therapeutic agents by alert observation in the laboratory or clinic. Well-known examples of this approach are the discovery of penicillin by Fleming and the recognition of the diuretic activity of organic mercurials employed in the treatment of syphilis. Undesired effects of older agents have also been exploited in the development of new drugs, as illustrated by the evolution of the carbonic anhydrase inhibitors, the antihypertensive thiazides, and the oral hypoglycemics from the antibacterial sulfonamides.

Development and Evaluation of New Drugs. The development and evaluation of new drugs in the United States is rigidly controlled by federal regulations administered by the Food and Drug Administration (*see* below). A new drug may not be marketed for general clinical use until it has been subjected to thorough clinical pharmacological studies, and until "substantial evidence" of its efficacy and safety has been obtained from adequate, well-controlled clinical trials conducted by qualified investigators.

Before initial studies in man are permitted, the full pharmacological spectrum of a new drug must be thoroughly and extensively explored in *animals*, and both acute and chronic toxicity tests must be conducted on several species. Because of species variations, such studies are considered useful merely as evidence that the drug has sufficient promise and is sufficiently safe to warrant testing in man. Even the most extensive studies in animals cannot substitute for successful clinical trials as evidence of clinical efficacy. The *initial clinical trials* of a new drug are necessarily cautious experiments, on volunteer normal subjects as well as patients, aimed primarily at establishing that the drug merits further study. If the initial trials in patients provide promise of clinical efficacy, the drug is subjected to thorough *clinical pharmacological studies*, and documentation of its efficacy and safety is sought in *controlled*

clinical trials. Only under exceptional circumstances may a new drug be administered to an individual without his informed consent.

What constitutes an adequately controlled clinical trial necessarily varies, depending upon the drug effect being evaluated. The more important general requirements for all trials are an appropriate and *sensitive method* of evaluation, an *adequate number of subjects, lack of bias, concurrent comparison* of the new drug with a *reference drug* over a *range of doses,* and appropriate *statistical validation.* Many clinical trials must be conducted under so-called *blind* conditions. In a blind experiment, the nature of the medication is concealed from the patient (single-blind) or from both the patient and all persons associated with conduct and evaluation of the trial (double-blind). Blind conditions are particularly essential for trials in which subjective effects of medication are being studied; they may also be necessary in evaluation of certain objective drug effects, if these are under voluntary control or otherwise easily biased. In addition to being compared with a reference drug, a new compound is often compared with inert *dummy* medication, to serve as a control for *placebo effects,* namely, those that are temporally correlated with administration of a drug but cannot be attributed to its pharmacological properties. Placebo effects result in part from the significance of the therapeutic effort to the patient and the set imparted at the time of drug administration. However, other coincident effects, such as spontaneous remission of symptoms, also contribute to the value of a placebo control.

In certain limited circumstances, a *placebo* may be administered in the course of regular therapeutic practice. If an inert lactose capsule or an injection of saline solution is employed for this purpose, it is termed a *pure placebo.* If a subeffective dose of a vitamin or other active drug is used, it is termed an *impure placebo,* since the resulting placebo effect may be erroneously attributed to pharmacological properties of the drug. The administration of a placebo in clinical practice is *not* a reliable procedure by which to distinguish between "psychogenic" and "somatic" disorders on the basis of whether relief of symptoms is obtained.

Because the capacity of existing facilities for conducting controlled clinical trials is limited, and for other practical reasons, controlled trials cannot possibly be conducted on all types of patients or under all varieties of clinical conditions. Consequently, evaluation of the efficacy of a drug may continue well into the period of its *general clinical use.* More important, since drug toxicity may occur only in a limited portion of the population, only after long chronic use, or only in combination with other variables, accurate assessment of the toxic potential of a new drug may not be possible until it has been in general use for several years.

Drug Regulations. There are a number of regulations and compendia concerned with the testing, labeling, purity, and quality of foods, drugs, and cosmetics. Such regulations are designed for the protection of the public health. The following regulations apply to the United States; other countries have similar codes.

Food and Drug Administration. The Federal Food, Drug, and Cosmetic Act of 1938 assures the quality and purity of drugs, by requiring accurate labeling of all medicinals. The initial law, the Federal Pure Food and Drugs Act, was passed by Congress in 1906, as a result of the excessive adulteration and misbrandings of foods and drugs existing at that time. The subsequent modifications of the act—in 1938 and 1962—were, unfortunately, a result of tragedies brought about by inadequate testing of new drugs or vehicles for drugs. Enforcement of the law is entrusted to the Food and Drug Administration (FDA) of the Department of Health, Education, and Welfare. Regulations resulting from the Drug Amendments Act of 1962 are concerned with establishing the efficacy as well as the safety of new drugs. In addition, they place responsibility with the FDA for continuing evaluation of the toxicity of drugs already in general use. Drugs found to be too dangerous in proportion to their therapeutic worth can be removed from the market. Another provision of the Drug Amendments Act was a retro-

spective evaluation of the efficacy of all drugs introduced from 1938 to 1962. This important and difficult review process was undertaken for the FDA by panels of experts organized by the Drug Research Board of the National Academy of Sciences–National Research Council.

Because of severe abuses of barbiturates, psychopharmacological agents, and other centrally acting drugs, the Comprehensive Drug Abuse Prevention and Control Act was passed in 1970. This gives the Bureau of Narcotics and Dangerous Drugs, Department of Justice, special powers to regulate the distribution of drugs liable to be abused.

Decisions as to whether drugs may be sold "over the counter" or dispensed only on prescription and the refilling of prescriptions are regulated by the FDA under the provisions incorporated in the Durham-Humphrey Amendment of 1952 (*see* Appendix). The FDA establishes special standards for insulin, antibiotics, and germicides and is responsible for certification of their safety and efficacy. The safety of food additives and the purity and quality of foods and cosmetics are also under the jurisdiction of the FDA. The purity and the efficacy of veterinary preparations are controlled as rigidly as are human medicinals.

The United States Pharmacopeia (U.S.P.) and The National Formulary (N.F.). The Federal Pure Food and Drugs Act of 1906 recognized the U.S.P. and the N.F. as "official compendia," thereby giving official status to the drugs and the standards set forth in these volumes. The approved therapeutic agents used in medical practice are described and defined with respect to source, chemistry, physical properties, tests for identity, tests for purity, assay, method of storage, and average therapeutic dosage. They were initially written as guides to the physician in his choice of drugs. However, because they serve as official standards for the quality and purity of drugs, these compendia are now of more use to the pharmaceutical industry and the FDA than to physicians. Nevertheless, the prescribing of *official* drugs listed in either the U.S.P. or N.F. provides assurance to the physician that the patient will receive exactly what has been prescribed with respect to quality and chemical uniformity. However, no tests of biological equivalence of official drugs are required, but they are under serious consideration. Other nations have similar compendia; there is also a *Pharmacopoea Internationalis* (sponsored by WHO), as well as a *European Pharmacopoeia* (1st ed., 1970).

U.S.P. Most of the preparations in the U.S.P. are single drugs. In the case of those that must be compounded, instructions are given for their preparation. The U.S.P. organization also provides reference standards for the assaying and testing of many of the U.S.P. drugs.

The first *Pharmacopeia* in the United States was published in 1820, and since that time numerous revisions have appeared. The current edition, U.S.P. XVIII, became official in 1970. The *Pharmacopeia* is revised at five-year intervals. The U.S.P. is revised by a special Pharmacopeial Committee, the members of which donate their services in the interest of the important function they serve. The committee consists of outstanding pharmacologists, physicians, and pharmacists.

N.F. This compendium was first published in 1888 under the name *National Formulary of Unofficial Preparations*. After acceptance as an official drug standard in 1906, the name was changed to its present title. The current edition is N.F. XIII, published in 1970.

The N.F. formerly contained drugs on the basis of demand as well as of therapeutic value, but beginning with N.F. XII therapeutic value was adopted as the sole criterion for the admission of drugs. Many drugs that have been deleted from the U.S.P. appear in the N.F. Unlike the U.S.P., it also contains formulas of certain drug mixtures. The N.F. is published by the American Pharmaceutical Association. It is prepared by a committee and advisory panels consisting mainly of outstanding pharmaceutical scientists and physicians.

Other Regulative Laws. The laws embodied in the *Harrison Narcotic Act* and the *Federal Marihuana Regulations* are superseded by the Comprehensive Drug Abuse Prevention and Control Act of 1970 and are enforced by the Bureau of Narcotics and Dangerous Drugs, U.S. Department of Justice; this act also includes other drugs subject to abuse. The law controls the distribution of opium, coca, cannabis, and any of their natural or synthetic derivatives, barbiturates, amphetamines, LSD, etc. (*see* Appendix). In addition, state and city laws exist to regulate the sale of narcotics, barbiturates, and similar drugs.

GUIDE TO THE "THERAPEUTIC JUNGLE"

The flood of new drugs in recent years has provided many dramatic improvements in therapy, but it has also created a number of problems of equal magnitude. Not the least of these is that of the "therapeutic jungle," the term used to refer to the combination of the overwhelming number of drugs, the confusion over nomenclature, and the associated uncertainty of the status of many of these drugs. A reduction in the marketing of close congeners and drug mixtures and an improvement in the quality of advertising are important ingredients in the remedy for the "therapeutic jungle." However, the physician can also contribute to the remedy by adopting a "way of thinking about drugs" based upon general pharmacological principles, by employing nonproprietary rather than proprietary names whenever possible, by using prototypes both as an instructional device and in clinical practice, by adopting a properly critical attitude toward new drugs, and by knowing and making use of reliable sources of information.

Drug Nomenclature. The existence of many names for each drug, even when reduced to a minimum, has led to a lamentable and confusing situation in drug nomenclature. In addition to its formal *chemical* name, a new drug is usually assigned a *code* name by the pharmaceutical manufacturer. If the drug appears promising, and the manufacturer wishes to place it on the market, a *United States Adopted Name* (USAN) is selected by the USAN Council, which is jointly sponsored by the American Medical Association, the American Pharmaceutical Association, and the United States Pharmacopeial Convention, Inc. (*see* Appendix). This *nonproprietary* name is often referred to as the *generic* name, but this latter term is properly reserved to designate a chemical or pharmacological class of drugs, such as sulfonamides or sympathomimetics. If the drug is eventually admitted to the U.S.P. or N.F., the USAN becomes the *official* name. However, the nonproprietary name and the official name of an older drug may differ. There is increasing worldwide adoption of the same name for each therapeutic substance. For newer drugs, the USAN is usually adopted for the nonproprietary name in other countries, but this is not true for older drugs. International agreement on the USAN name is mediated through the World Health Organization and the pertinent health agencies of the cooperating countries. Subsequently, the drug will also be assigned a *proprietary* name or *trademark* by the manufacturer. If the drug is marketed by more than one company, it may have several proprietary names. If mixtures of the drug with other agents are marketed, each such mixture may also have a separate proprietary name.

To minimize confusion, the nonproprietary or official name of a drug should be used whenever possible, and this practice is adopted in this textbook. The question arises, however, whether the nonproprietary name or a proprietary name should be used by the physician when prescribing drugs. The use of the nonproprietary name is clearly less confusing when the drug is available under multiple proprietary names. However, an important problem concerns the distinction between chemical, biological, and clinical equivalence of drugs. *Chemical equivalents* are identical dosage forms that contain identical amounts of the same chemical substance and meet the physicochemical standards in the official compendia. *Biological equivalents* are those chemical equivalants that, when administered in the same amounts, provide the same biological or physiological availability, as measured by plasma or tissue levels. *Clinical equivalents* are those chemical equivalents that, when administered in the same amounts, provide the same therapeutic effects, as measured by the control of a symptom or a disease. The clinical equivalence of commercial products has been the center of heated controversy.

The main question has been whether drugs that are chemical equivalents are, in fact, clinical equivalents. This is important, since drugs marketed by different companies under the nonproprietary name may differ markedly in cost from the same drug marketed under the proprietary name. Since clinical equivalence of drugs is difficult to quantify because of marked individual differences and of vari-

ations in the symptoms or disease being studied in man, attention has been directed to assessment of the biological equivalence of drugs. This is based on the sound premise that equal tissue or plasma levels of two different preparations of the same drug will give the same therapeutic effect. Tests of biological equivalence of preparations from various companies have demonstrated that differences do occur with drugs that are administered orally in solid dosage forms, such as tablets and capsules. These differences are due to variations in formulation factors, such as particle size, crystal form, adjuvants, fillers, binders, and pressures used to compress tablets, all of which affect the dissolution rate, an important factor in oral absorption of drugs. Even preparations of the same drug with dissolution rates within official standards may not be biological equivalents. Unfortunately, since the majority of medicinals have not been evaluated for biological equivalence, the magnitude of the problem cannot be stated. However, it would be expected to be much less important for highly soluble drugs and even for poorly soluble drugs that are formulated under well-controlled conditions and that meet rigid standards. Until biological and clinical equivalence of medicinals can be assured, the physician is well advised to prescribe only preparations of high quality. *It is, therefore, recommended that drugs be prescribed by nonproprietary name in all cases. However, when a particular dosage form or special formulation of a drug is desired, and especially when accurate control of dosage is critical, the prescription order should then include, in addition to the nonproprietary name, the name of the manufacturer of the preparation desired* (*see* Appendix).

Use of Prototypes. For teaching purposes, as illustrated in this textbook, the confusion created by the welter of similar drugs is reduced by restricting major attention to prototypes in each pharmacological class. Focusing on the representative drugs results in better characterization of a class as a whole, and thereby permits sharper recognition of the occasional member that possesses unique properties. A teaching prototype is often the agent of choice, but this is not always true. A particular drug may be retained as the prototype, even though a new congener is clinically superior, either because more is known about the older drug or because it is more illustrative for the entire class of agents.

The clinician will also find the prototype device helpful in his struggle with the surfeit of congeneric drugs, since his needs for therapeutic agents can usually be adequately satisfied by one or two drugs in each class. Which of a number of more-or-less equivalent drugs the physician actually chooses as his prototypes may be determined by differences in their duration of action or other secondary characteristics. The important consideration is that he restrict his attention to a limited number of drugs in each class and that he become thoroughly familiar with their individual characteristics. If he does, he will inevitably use these agents more effectively than he would if he were to change repeatedly among a larger number of drugs. Moreover, the greater experience with a few drugs will provide a better base line of personal experience by which he may judge the claims for newer medicinals.

Attitude toward New Drugs. A reasonable attitude toward new drugs is summarized by the adage that advises the physician to be "neither the first to use a new drug nor the last to discard the old." This advice is intended as a reminder that only a minor fraction of the new drugs released each year represents significant therapeutic advance, and that the efficacy and safety of a new drug, particularly relative to older agents, may not be fully assessed until sometime after it has been in general clinical use. It also stresses the physician's obligation to keep abreast of significant advances in pharmacotherapy. However, appropriate, timely change from the old drug to the new is possible only if the physician has access to prompt, unbiased, critical information about new drugs.

Information about Drugs. *Pharmacology textbooks* usually provide basic pharmacological principles, critical appraisal of the therapeutically useful classes of drugs, and

detailed descriptions of the prototypes that serve as standards of reference for assessing new drugs. However, for obvious reasons, these textbooks cannot include information on the most recently introduced drugs, nor can they provide detailed descriptions of many of the older drugs. *Remington's Pharmaceutical Sciences*, the *United States Dispensatory and Physicians' Pharmacology*, the *American Hospital Formulary Service*, and various drug encyclopedias are sources of information about drugs; historical accounts of older drugs are found in the older editions. The *Physicians' Desk Reference (PDR)* is a convenient source of information about available products, dosage forms, contraindications, untoward reactions, and allowable therapeutic claims, but it is *not* useful as a critical guide to therapy or as a source of pharmacological effects of drugs. Legally, all information on drugs described in *PDR* must conform to the descriptive material in the package insert. The information, in turn, must meet the approval of the FDA. The *American Drug Index* is also a useful cross-indexed source of drug products and dosage forms, listed by both nonproperietary and proprietary names.

Somewhat more current information about drugs is provided by *AMA Drug Evaluations (ADE)*, a publication of the Council on Drugs of the American Medical Association. The Council previously published several annual indices of drugs, which were sequentially named *New and Nonofficial Remedies*, *New and Nonofficial Drugs*, and *New Drugs*. *ADE* is a useful and authoritative reference book that evaluates information on both old and new single-entity drugs and mixtures, arranged according to therapeutic category. It is designed to help the physician in the selection and use of drugs. A very useful source of information on new drugs is the biweekly publication, *The Medical Letter on Drugs and Therapeutics*. Its distinguished board of editors provide a distinct service to medicine and to physicians by furnishing prompt, unbiased, pointed assessment of new drugs. A British publication, the *Drug and Therapeutics Bulletin*, is also published fortnightly with the same high standards. The *Prescribers' Journal*, published bimonthly by the British Ministry of Health, also contains objective and critical analyses of new therapeutic agents. *Pharmacology for Physicians* is a monthly publication in which experts discuss the clinical pharmacology of drugs used for the therapy of particular diseases. Other current and critical sources of information on drugs, drug therapy, and drug toxicity include the periodic appraisals sponsored by the Council on Drugs and published in the *Journal of the American Medical Association*, and the editorials and short reviews in *Clinical Pharmacology and Therapeutics*, the *New England Journal of Medicine*, and similar periodicals. *Pharmacological Reviews* and *Annual Review of Pharmacology* are excellent sources of basic pharmacological information.

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In vitro and *in vivo* pharmacologic activities of antisense oligonucleotides

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Summary: The use of antisense oligonucleotide as pharmacologic agents is a derivative of the central dogma of molecular biology and knowledge of the physical and chemical properties that govern the structure of nucleic acids. Oligonucleotides have been reported to inhibit the growth of a large number of viruses in cell culture, as well as the expression of numerous ocogenes, a variety of normal genes and transfected reporter genes controlled by several regulatory elements. The therapeutic activity of antisense compounds in animal disease models have also been reported.

This review provides some general conclusions and trends regarding the pharmacologic action of antisense oligonucleotides, that can be formulated from studies previously reported in the literature. In addition, data is highlighted for two specific examples in which antisense oligonucleotides have demonstrated activity against herpes viruses and intracellular adhesion molecule RNA targets.

Introduction

In the past few years, many papers have been published demonstrating the activity of numerous antisense oligonucleotides, of different sequences and chemical type, in a variety of cell-based systems. Recently there have been a number of excellent reviews that have summarized the activities of these compounds in detail (Cohn, 1989; Uhlman & Peyman, 1990; Cazenave & Helene, 1991). As such this review will not attempt to duplicate those comprehensive efforts; instead it will provide a brief summary of the activities of oligonucleotides in cell-based assays and attempt to provide some general conclusions and trends that can be formulated from these previously published data. In addition, this paper will provide examples of data compiled in our laboratories that relate to the pharmacological activities of phosphorothioate oligonucleotides directed against cellular and infectious disease targets.

Pharmacological activities in cell-based models

Oligonucleotides have been reported to inhibit the growth of a large number of viruses in cell culture, as well as the expression of numerous oncogenes, a variety of normal genes and transfected reporter genes controlled by several regulatory elements. These studies varied in the types of oligonucleotides used, the cells used, the RNAs and specific receptor sequences targeted and the conditions employed. Although a wide range of oligonucleotide concentrations have been used to treat cells, only a few studies have reported detailed dose-response curves and clearly documented the purity of the oligonucleotides used. Table I summarizes the information from more than 40 papers in which oligonucleotides were tested for pharmacologic

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Table I Summary of cell-based *in vitro* activities of antisense oligonucleotides

Target viruses	*Cell type*	*Serum*	*Oligo types*	*Oligo length*	*Effective oligo concentration*	*References*
HTLV-III	H9 cells	–	P	12–26	5–50 mg/ml	Zamecnik *et al.* [1986]
HIV	H-T cells	+	P-S	14–28	0.5 μM	Matsukura *et al.* [1987]
HIV (gag/pol)	H-T cells	+	P-S	18–24	1–10 μM	Kinchington & Galpin [1989]
HIV	H9 cells	+	PS, others	20	4–20 μg/ml	Agrawal *et al.* [1988]
HIV	CZM cells	+	PS	18–28	10 μM	Vickers *et al.* [1991]
Herpes simplex	Vero cells	+	CH3-P	7	50–100 μM	Smith *et al.* [1989]
Herpes simplex	HeLa cells	+	PS	28	1–10 μM (non-antisense)	Gao *et al.* [1989]
Herpes simplex	Vero cells	+	CH3-P	12	20–50 μM	Kulka *et al.* [1989]
Herpes simplex	Vero cells	+	CH3-P-psoralen	12	5 μM	Kulka *et al.* [1989]
Herpes simplex	HeLa cells	+	PS	21	0.2–4 μM	Draper & Brown-Driver [1991]
Vesicular stomatitis	L929 cells	+	CH3-P	9	25–50 μM	Agris *et al.* [1986]
Vesicular stomatitis	L929 cells	+	P-lipid	11	50–150 μM	Shea *et al.* [1991]
Vesicular stomatitis	L929 cells	+	p-poly l-lysine	10–15	0.1 μM	LeMaite *et al.* [1987]
Influenza	MDCK cells	+	P-acridine	11	50 μM	Zerial *et al.* [1987]
Tick borne encephalitis	–	+	Various	Various	0.1–1 μM	Vlassov [1989]
SV40	MDCK cells	+	CH3-P	6–9	25 μM	Miller *et al.* [1985]
Rous	Chicken fibroblasts	+	Various	Various	10 μM	Zamecnik & Stephenson [1978]
Hepatitis B	Alexander	+	P	15	8.5 μM	Goodarzi *et al.* [1990]
Bovine papilloma virus	C-127 cells	+	PS	4–30	0.01–1 μM	Cowsert & Fox [1991]

Oncogenes						
c-myc	T-lymphocytes	+	P	15	30 μM	Heikkila *et al.* [1987]
c-myc	HL-60 cells	+	P, PS	15	10 μM	Wickstrom *et al.* [1989]
c-myc	Burkitt cells	–	P	21	100 μM	McMananway *et al.* [1990]
c-myb	PMBC	+	P	18	40 mg/ml	Gewirtz *et al.* [1989]
BCL-2	L697 cells	–	P ± PS	20	25–150 μM	Reed *et al.* [1990]
N-ras	T15 cells	+	CH3-P	9	Inactive	Tidd *et al.* [1988]
Host genes						
Multiple drug resistance	MCF-1 cells	+	PS	15	—	Jaroszewski *et al.* [1990]
PCNA (cyclin)	3T3	+	P	18	30 μM	Jaskulski *et al.* [1989]
Prothymosin	Human myeloma cells	–	P	16–21	40 μM	Sburlati *et al.* [1991]
T cell receptor	T cells	+	P	22	—	Zheng *et al.* [1989]
Gm CSF	Endothelial cells	–	P	15, 18	10^{-5} M	Zheng *et al.* [1989]
CSF-1	FL-ras/myc cells	+	P	—	—	Birchenall *et al.* [1989]
EGF receptor		+	P	13	30 μM	Yeoman *et al.* [1989]
β Globin	Rabbit reticulocytes	+	CH3P	9	100 μM	Blake *et al.* [1985]
cAMP-protein kinase II_{β}	HL-60	+	P	21	15 μM	Tortora *et al.* [1990]
Phospholipase A2 Activating protein	BC3H1	+	P	25	25 μM	Clark *et al.* [1991]
bFGF	Human astrocytes	–	P	15	10–75 μM	Morrison [1991]
TAU	Neurons	–	P	20–25	3–50 μM	Caceres & Kosik [1990]
Myeloblastin	HL-60 cells	+	P	18	—	Bories *et al.* [1989]
ICAM-1	A549, HUVEC	–	PS	18–20	0.01–1 μM	Chiang *et al.* [1991]
IL-2	T lymphocytes	–	P	15	5 μM	Harel-Bellan *et al.* [1988]
IL-1α	HUVEC	+	P	18	10 μM	Maier *et al.* [1990]
IL-1β	Monocytes	+	PS	15	0.1–2.5 μM	Manson *et al.* [1990]
IGF-1	Myoblasts	–	P	15	10 μM	Florini & Ewton [1990]
Perforin	T lymphocytes	–	P	18	5–35 μM	Acha-Orbea *et al.* [1990]

Table I *(contd.)*

Target	*Cell type*	*Serum*	*Oligo types*	*Oligo length*	*Effective oligo concentration*	*References*
Other						
Chloramphenicol acetyl transferase	CV-1 cells	+	P, PS, CH3P	21	5–30 μM	Marcus-Sekura *et al.* [1987]
Placental alkaline Phosphatase driven by HIV TAR	SK-mel-2 cells	+	PS	18–28	0.25–5 μM	Vickers *et al.* [1991]
Chloramphenicol acetyl transferase driven by human papilloma virus E2 responsive element	C-127 and CV-1 cells	+	PS	14–20	1–10 μM	Cowsert & Fox [1991]

cAMP = cyclic AMP; EGF = epidermal growth factor; G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte macrophage colony-stimulating factor; HB = hepatitis B; HIV = human immunodeficiency virus; HSV = herpes simplex virus; HTLV = human T cell lymphotrophic virus; IV = influenza virus; PCNA = proliferating cell nuclear antigen; RSV = Rous sarcoma virus; TAR = TAT response element; TBE = tick-borne encephalitis; VSV = vesicular stomatitis

activities against a variety of viruses, oncogenes, host genes and transfected reporter genes.

The data presented in Table I support only a few generalizations. First, while phosphodiesters are rapidly degraded in biological systems, a number of investigators have reported activities for unmodified phosphodiester oligonucleotides in cells incubated in the absence of serum or in medium supplemented with heat-inactivated serum. When phosphodiester oligonucleotides have displayed activity, concentrations of more than 10 μM were required. The explanation for these activities is unclear. Considering the presence of endo- and exonucleases that are found within cells it is reasonable to think that these oligonucleotides would be degraded in the cell very rapidly. Evidence from our laboratory demonstrates that in a number of routinely used cell lines phosphodiester oligonucleotides are degraded within minutes by nucleases found in the plasma membrane, cytoplasm and in nuclei (Hoke et *al.*, in press).

Second, a variety of chemically modified oligonucleotides have been reported to be active in cell culture. Although considerable variation has been reported, phosphorothioate oligonucleotides appear to be more potent than methylphosphonate oligonucleotides. Conjugation of alkylators and interchelators to phosphodiesters and methylphosphonates has been reported to increase potency. Many of these modifications have been positioned at either the 3' or 5' end of the oligonucleotides; 3' positioning is an attempt to increase stability to 3'-exonuclease, the predominant serum nuclease. Lipophilic and poly(L-lysine) conjugates have also displayed enhanced potencies presumably via some modulation of cellular pharmacokinetic characteristics.

Third, oligonucleotides have demonstrated activities against a broad array of viral targets, oncogenes, normal cellular gene products and various transfected genes. This array of pharmacological effects clearly demonstrates the broad potential therapeutic applicability of these drugs.

Fourth, although the data from studies included in Table I are limited, when it is combined with in vitro toxicologic data (Crooke, 1991), the therapeutic indexes of phosphorothioate oligonucleotides appear to be quite high. Initial data regarding certain phosphorothioates of 20 and 21 nucleosides in length, targeted to human papilloma virus and herpes simplex virus, respectively, also demonstrate that these compounds are extremely well tolerated in animals (Mirabelli et *al.*, in preparation). The effects of specific base composition within an oligonucleotide, oligonucleotide length, specific chemical modifications in oligonucleotide and cellular parameters (i.e. cell type, cell cycle phase and stages of differentiation) on the potential toxicology and non-antisense activities of these compounds are not yet clearly defined (Crooke, 1991).

Fifth, very little data that support putative mechanisms of action have been reported and generalizations concerning precise mechanisms of action are not possible. A variety of mechanisms have been proposed to explain the ultimate pharmacologic action of antisense oligonucleotides, all resulting from the hybridization of the drug with the complementary sequence within a target RNA. These mechanisms include the disruption of ribosomal assembly and function, formation of an RNase H substrate and subsequent cleavage of the target RNA, and disruption of RNA splicing processes or other RNA metabolic processes. It is very likely that many 'terminating' mechanisms can be exploited for the cellular action of antisense oligonucleotides and that the mechanisms of a particular oligonucleotide are the result of the particular RNA and sequence target, the cell in which the drug is. acting and the chemical structure of the oligonucleotides.

Examples of antisense pharmacologic activities

Our laboratory has demonstrated activities of oligonucleotide drugs against a number of molecular disease targets. Below is a brief summary of work on two targets: herpes simplex virus and a human cell adhesion molecule, ICAM-1. These data are reviewed in an attempt to provide examples of the antisense drug discovery process and the activities of antisense compounds directed against viral gene targets and host gene targets.

Antisense oligonucleotides directed to herpes simplex virus RNA targets

In vitro activities. Smith & Smith (1986) first reported antisense inhibition of HSV replication using oligonucleotides targeted to the splice junction sequences of the HSV-1 1E4 and 1E5 pre-RNAs. It was later reported that increasing the length of the oligonucleotide increased the antiviral activity against HSV-1 (Kulka *et al., 1989).* The most active oligonucleotide, a 12-nucleotide long oligomethylphosphonate, was directed against a splice junction covering six nucleotides in both exon and intron. The potency of the compound was greatest when added at the time of infection (IC_{50} = 15 μM) with a 5- to lo-fold reduction in potency when the oligonucleotide was added 1 h post-infection. A 20% inhibition in splicing was observed in oligonucleotide treated infected cells *versus* untreated infected cells. Conjugation of the 12-mer oligomethylphosphonate with a psoralen-derivative increased the potency of the compound approximately 3-fold relative to the unconjugated compound. However, the psoralen conjugate required activation by UV irradiation following addition to the infected cells.

A study by Draper *et al.* (1990) using phosphodiester oligonucleotides complementary to two related region of the HSV-1 Vmw 65 mRNA, reported that an oligonucleotide targeted to the translation initiation region effectively inhibited HSV-1 replication. The other oligonucleotide was inactive, causing these authors to conclude that sequences within the same mRNA can exhibit differential sensitivities to antisense oligonucleotides.

Our laboratory has designed and tested several oligonucleotides which are complementary to the translation initiation regions of several mRNAs of HSV. Oligonucleotides which target the HSV UL13 mRNA were found to be effective inhibitors of HSV replication, as measured in an infectious yield assay (Draper & Brown-Driver, 1991; Draper *et al.,* submitted). The protein encoded by the UL13 gene has been putatively identified as a phosphotransferase which may be involved in the phosphorylation of viral capsid proteins (Smith *et al.,* 1986; Stevely *et al.,* 1985). Preliminary screening experiments revealed that phosphorothioate oligonucleotides were significantly more potent than phosphodiester and methylphosphonate oligonucleotides (Draper & Brown-Driver, 1991; Draper *et al.,* submitted). One of the most potent compounds evaluated was ISIS 1082, a 21-mer phosphorothioate oligonucleotide, targeted to a secondary initiation codon present in HSV-1 and HSV-2 UL13 mRNA. This compound inhibited both HSV-1 (KOS strain) and HSV-2 (HG52 strain) replication in an infectious yield assay. Site specific cleavage of synthetic UL13 transcripts was induced by addition of ISIS 1082 in RNA processing extracts of HeLa cells suggesting that ISIS 1082 may inhibit expression of the UL13 gene product by inducing RNAase H specific cleavage of UL13 mRNA.

Evaluation of the compound in infectious yield assays using acyclovir sensitive and resistant strains and in comparative dose responses with acyclovir and other phos-

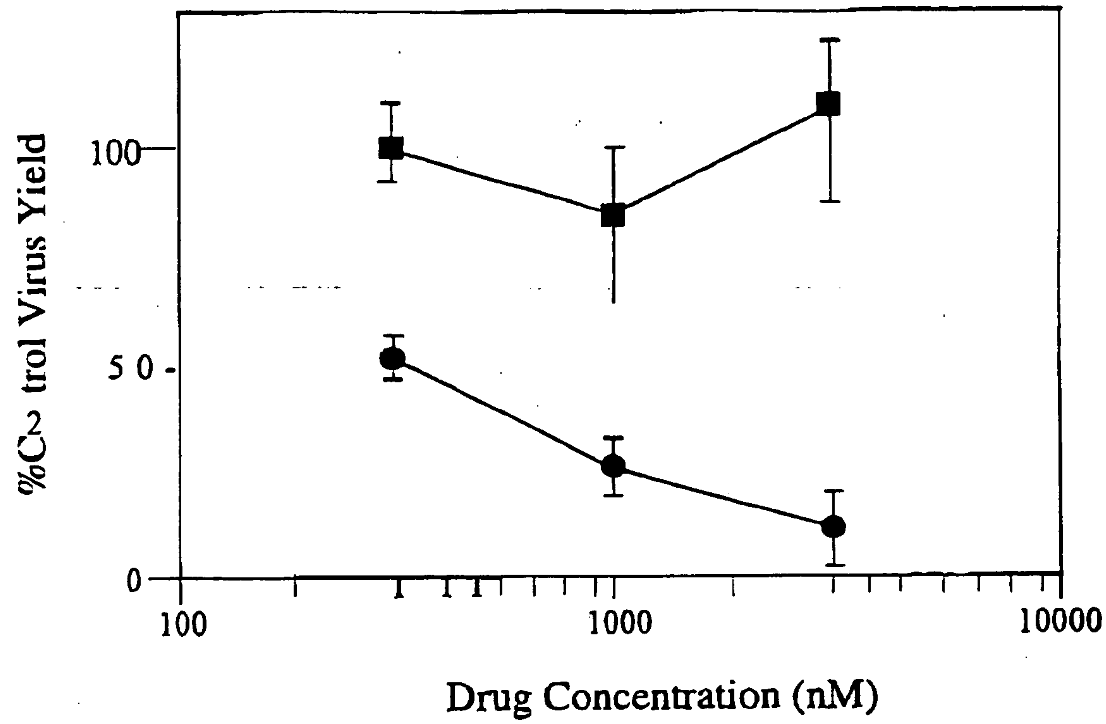

Figure 1 Sensitivity of an acyclovir resistant strain of HSV-1 (DM 2.1, thymidine kinase deletion mutant) to ISIS 1082 (●) and acyclovir (■). Activities were measured in an infectious yield assay and expressed as a percent of untreated infected cell virus yield

phorothioate oligonucleotides provided more evidence that ISIS 1082 produces its antiviral activity via a sequence specific antisense effect. First, ISIS 1082 inhibits the replication of the HSV-1 strain KOS in HeLa cells by 50% and 90% at concentrations of approximately 300 nM and 2 μM, respectively. ISIS 1082 was 3- to lo-fold more potent then a phosphorothioate oligonucleotide of similar length and equivalent (but scrambled) nucleotide base composition when tested against certain strains of HSV-1 and HSV-2. In addition, it was found that ISIS 1082 was active against a number of acyclovir resistant strains of HSV-1. Figure 1 shows the activity of ISIS 1082 against the thymidine kinase deletion mutant strain, DM2.1. Acyclovir did not inhibit the replication of this strain. However, treatment with ISIS 1082 resulted in a dose-dependent decrease in infectious viral yield that was equivalent to that observed against the KOS strain of HSV-1. At concentrations as high as 100 μM of ISIS 1082, only minimal effects on host cell growth and metabolism were observed (Crooke *et al.*). This lack of *in vitro* toxicity is again consistent with the postulated highly selective mode of action of the antisense compound.

In vivo activities. Earlier preliminary reports have suggested *in vivo* activities of antisense drugs against HSV infections. One report indicated that an oligomethylphosphonate was active in a mouse model of herpes simplex virus 1 infection (Kulka *et al.*, 1989). Two additional laboratories have reported on the activity of phosphorothioates against HSV-1 infections in mouse models of ocular herpetic keratitis (Kimura *et al.*, 1991; Metcalf *et al.*, 1991).

Recent data have demonstrated that topical application of ISIS 1082 in an aqueous buffer to the cornea of mice infected with HSV-1 (KOS) resulted in curative activity at drug concentrations of 0.3% and 5% (Brandt *et al.,* 1991; Brandt *et al.* submitted). The activity of ISIS 1082 in this model was equivalent to trifluorothymidine and exhibited no local or systemic toxicities. ISIS 1082 is currently being studied in rabbit models of HSV-1 induced epithelial keratitis and other animal models of dermal and systemic HSV infection to better define the pharmacology of the compound.

Antisense oligonucleotides directed to intercellular adhesion molecules

To date most reports of antisense oligonucleotide activities in non-viral infection models have focused on oncogene targets and receptor signaling targets as seen in Table I. Our laboratory has recently explored the use of antisense oligonucleotides to pharmacologically manipulate the expression of certain cellular adhesion molecules (Chiang *et al.,* 1991).

Rationale for adhesion molecules as antisense targets

The binding of circulating leukocytes to vascular endothelium is an obligatory step in the emigration of leukocytes out of the vasculature to the site of infection or injury (Harlan, 1985). Several endothelial proteins have recently been identified which mediate the adherence of leukocytes to inflamed vascular endothelium and subsequent migration out of the vasculature (Stoolman, 1989; Osborn, 1990; Springer, 1990). One such protein, ICAM-1, is a 95-105 kD glycoprotein first identified by the ability of a monoclonal antibody to block phorbol ester induced aggregation of a B-cell line (Rothlein *et al.,* 1988). The cellular distribution of ICAM-1 is different from other endothelial cell adhesion molecules in that it is expressed in both endothelial cell and non-endothelial cells including leukocytes, fibroblasts, kernatinocytes and other epithelial cells (Table II). ICAM-1 binds circulating leukocytes through LFA-1 (CD11a, CD18), a member of the β_2 integrin family (Marlin & Springer, 1987). ICAM-1 is a member of the immunoglobin gene superfamily containing five immunoglobulin domains (Simmons *et al.,* 1988; Staunton *et al.,* 1988; Tomassini *et al.,* 1989). Expression of ICAM-1 is inducible by a number of cytokines including IL-1, TNF-α and IFN-γ (Rothlein *et al.,* 1988; Stoolman, 1989; Osborn, 1990; Springer, 1990). The broad tissue distribution of ICAM-1 suggests that it is not only involved in the emigration of leukocytes out of the vasculature, but may play a more extensive role in immune responses. Additional roles suggested for ICAM-1 include localization of leukocytes to the area of inflammation in extravascular spaces, enhancement of the recognition of antigen presenting cells by T lymphocytes, formation of lymphocyte germinal centers, enhancement of natural killer cell response and differentiation of thymocytes (Rothlein *et al.,* 1986; Dustin *et al.,* 1986 & 1988; Makgoba *et al.,* 1988; Altmann *et al.,* 1989; Boyd, 1989; Robertson *et al.,* 1990; Springer, 1990). In addition ICAM-1 is the receptor for over 90% of the rhinovirus serotypes (Staunton *et al.,* 1989; Tomassini *et al.,* 1989).

In vitro inhibition of ICAM-1 expression by antisense oligonucleotides

During the initial evaluation of a series of phosphorothioate oligonucleotides targeted to specific sites within the ICAM-1 mRNA it was found that the cationic lipid,

Table II Leukocyte adhesion molecules

Endothelial CAM	*Expressed on other cells*	*Gene family*	*Induction kinetics*	*Leukocyte ligand*	*Type of leukocyte bound*
ICAM-1	Keratinocytes, fibroblasts, leukocytes, etc.	Immunoglobulin	4 h to 72 h	LFA-1, MAC-1	Lymphocytes, monocytes, granulocytes
ICAM-2	Activated lymphocytes	Immunoglobulin	Constitutively	LFA-1	Lymphocytes, monocytes, granulocytes
VCAM-1	No	Immunoglobulin	4 h to 72 h	VLA4	Lymphocytes, monocytes
ELAM-1	No	LEC-CAM	2 h to 18 h	Carbohydrate	Granulocytes monocytes, memory T cells
GMP-140	Platelets	LEC-CAM	5 min to 2 h	Carbohydrate	Granulocytes, monocytes

DOTMA markedly enhanced the activity of the antisense oligonucleotides used in this study. DOTMA was originally described as a vehicle for transfection of DNA into cells (Felgner *et al.,* 1987). Cationic lipid delivery methods differ from normal liposomal delivery methods, in that the DNA or oligonucleotide is not encapsulated within the liposome, but rather is associated with the surface of the liposome through ionic interactions. Preliminary data in certain cell lines indicate that DOTMA enhances cell association of oligonucleotides at least lo-fold and markedly changes the intracellular distribution of the oligonucleotide, with apparently less oligonucleotide being concentrated in endosomes or lysosomes and more found in the nucleus (Chiang *et al.,* 1991; Bennett *et al.,* in preparation). Therefore, in some cells DOTMA will enhance oligonucleotide entry into the cytoplasm of cells similar to direct microinjection. The use of DOTMA has the advantage over microinjection experiments in that oligonucleotides can be introduced into large number of cells allowing biochemical analysis to be performed. In addition, it was determined that DOTMA had no effect on the expression of ICAM-1 when used at concentrations that maximized oligonucleotide uptake and activity (Chiang *et al.,* 1991). The use of DOTMA in these experiments allowed us to determine which regions on the ICAM-1 mRNA serve as the best target sites for antisense oligonucleotides and determined the mechanism by which antisense oligonucleotides inhibit ICAM-1 expression. To our knowledge this is the first report demonstrating that cationic lipids enhance antisense oligonucleotide activity in mammalian cells.

Using DOTMA as a formulation medium we have demonstrated that antisense oligonucleotides which target human ICAM-1 mRNA inhibit the expression of ICAM-1 in two cell culture systems HUVEC and a human lung carcinoma, A549 (Chiang *et al.,* 1991). Screening antisense oligonucleotides which target a number of sites on the ICAM-1 mRNA revealed that two sites were especially sensitive to inhibition with antisense oligonucleotides; the AUG translation initiation codon and specific sequences in the 3'-untranslated region. Data from these studies suggest that hybridization affinity is important for antisense oligonucleotides, as truncated versions of active oligonucleotides (<20-mers) exhibit decreased activity, however, hybridization affinity is not sufficient to ensure antisense activity. Therefore, target site selection is also an important parameter to consider when designing antisense oligonucleotides.

The most active ICAM-1 antisense oligonucleotide targets the 3'-untranslated region of the ICAM-1 mRNA. ISIS 1939 hybridizes to the ICAM-1 mRNA, nearly 300 bases 3'- to the translation termination site, therefore it should not directly affect translation of the protein. This oligonucleotide was shown to inhibit the expression of ICAM-1 in endothelial cells as measured by ELISA using a monoclonal antibody to ICAM-1 (Figure 2). Under equivalent experimental conditions treatment of endothelial cells with ISIS 1939 blocked the adhesion of HL60 cells. Thus the blockade of ICAM-1 expression was coincident with the loss of functional activity of the protein. Oligonucleotides which hybridized to other sequences in the 3'-untranslated region of ICAM-1 mRNA were not as effective as ISIS 1939 (Figure 2). Therefore, the effects of ISIS 1939 are unique to the target site to which it hybridizes.

ICAM-1 mRNA contains three repeats of a consensus sequence, AUUUA, thought to be involved in destabilization of mRNA (Caput *et al.,* 1986; Shaw & Kamen, 1986; Brawerman, 1989). An oligonucleotide that targets those sequences was shown to exhibit weak activity. However, ISIS 1939 targets an area approximately 200 bases 5'- to the AUUUA sequences. The region targeted by ISIS 1939 is predicted to be a stable stem loop structure which when bound would disrupt the structure. Analysis of steady state mRNA levels from oligonucleotide treated cells revealed that ISIS 1939

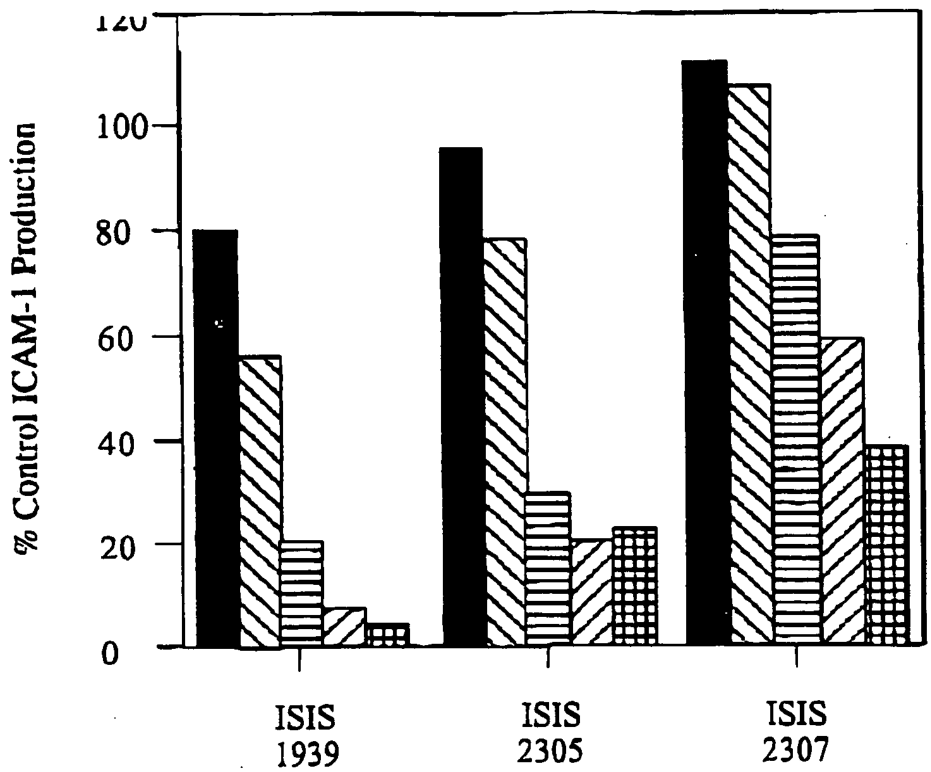

Figure2 Inhibition of IL-1 induced ICAM-1 expression in AS49 cells with antisense oligonucleotides which hybridize to the 3'-untranslated region of ICAM-1 mRNA. Cells were treated with phosphorothioate oligonucleotides (20 mers) at concentrations of ■ 0.1 μM ◻ 0.3 μM ◻ 0.5 μM ◻ 0.7 μM ▦ 1.0 μM in the presence of DOTMA. ICAM-1 expression was measured by ELISA using an ICAM-1 monoclonal antibody 84H10

specifically reduced the quantity of ICAM-1 mRNA per cell. The reduction of ICAM-1 mRNA was not due to decreased transcription of the ICAM-1 gene as analysed by nuclear run-off reactions. Therefore, ISIS 1939 must destabilize the ICAM-1 mRNA either by an RNase H dependent mechanism and/or by modulating natural processes which help to stabilize the ICAM-1 mRNA.

Oligonucleotides targeted to certain other specific sites within ICAM-1 mRNA were found to be potent inhibitors of ICAM-1 protein expression and cell adhesion. These oligonucleotides were targeted to sequences within the 5' untranslated region and the translation initiation region. The oligonucleotide targeted to the translation initiation region did not cause a reduction in the steady state level of ICAM-1 mRNA; unlike that found with ISIS 1939. Taken together these data suggest that different oligonucleotides targeted to different sites on an RNA may inhibit the production of a protein by different mechanisms.

Summary

The notion of using antisense oligonucleotides as pharmacologic agents is a derivative of the central dogma of molecular biology and knowledge of the physical and

chemical properties that govern the structure of nucleic acids. The practical evidence that antisense oligonucleotides can be drugs is a result of the work of a number of laboratories, including those cited in this review.

Key to the continued progress in the field of antisense therapeutics is the realization that oligonucleotides and their RNA targets work via the same principles of pharmacology that govern the actions of all other classes of drugs. Considering the properties of drugs that define their pharmacologic value, such as ligand-receptor binding affinity and fidelity and realizing the intrinsic properties of oligonucleotides, it is very clear that these compounds have enormous potential value in treating human diseases.

During the next few years a number of oligonucleotide compounds will enter into clinical trials. These first generation antisense drugs (e.g. phosphorothioates) will encounter many of the same issues and hurdles that confront all novel pharmaceutical agents; large-scale process development, adequate methods and tools to define clinical pharmacokinetics and metabolism, etc. Another important component of this process is the continued examination and definition of the molecular pharmacodynamics and pharmacokinetics of these drugs. We need to better understand how the structure and function of RNA defines the sensitivity of specific target sites to antisense oligonucleotides, the precise role of RNase H and other intracellular enzymes and proteins in the mechanism of action in oligonucleotides, the process by which oligonucleotides penetrate cellular membranes and distribute in cells, the non-sequence specific interactions that oligonucleotides can engage in both in and out of cells, and the metabolic pathways (both nuclease and non-nuclease) and metabolites that are likely to play a role in the metabolism of antisense drugs. The combination of this molecular, cellular, and clinical information will allow us to better determine the specific molecular targets and diseases that can be successfully treated with the first generation of antisense drugs. As important, it will define the biology, chemistry, and pharmacology of second and third generation antisense drugs.

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THERAPEUTIC APPLICATIONS OF OLIGONUCLEOTIDES

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INTRODUCTION

First proposed in 1978 by Zamencik & Stephenson (1), oligonucleotide therapeutics represent a new paradigm for drug discovery. The technology focuses on a class of chemicals, oligonucleotides, that has not been studied as potential drugs before and employs them to intervene in biological processes that likewise have not been studied previously as sites at which drugs might act.

The paradigm has resulted in substantial enthusiasm because oligonucleotides may display dramatic increases in affinity and selectivity for their nucleic acid targets compared to traditional drugs. Furthermore, antisense technology may facilitate rational drug design. Table 1 compares affinities and the potential for selectivity of oligonucleotides versus traditional drugs. The comparison is based on average affinities of typical traditional drugs in optimized assays with purified receptors and data derived from a 21-mer phosphorothioate oligonucleotide in binding assays performed in 1M NaCl. Hybridization varies substantially as a function of ionic strength, and the affinities at 100 mm NaCl in the presence of Mg^{2+} for the 21-mer are significantly lower. Furthermore, affinities may be lower in physiological systems with RNA that has secondary structure, so these comparisons present the opportunity in its broadest dimensions.

A number of terms have been coined and often misused to describe various

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Table 1 Affinity and selectivity of traditional and oligonucleotide drugs. Affinity constants were determined as described in text

Traditional drug		Oligonucleotide drug (1M NaCl)	
Affinity for receptor	10^{9}	Affinity for receptor sequence	10^{29}
Affinity for isotype	$10^{6}-10^{8}$	Affinity for one base mismatch	10^{26}
Maximum affinity for other proteins	$10^{6}-10^{8}$	Maximum affinity for nucleic acid binding proteins	$10^{12}-10^{13}$
ΔKd	$1-10^{3}$	Δ kD	$10^{4}-10^{11}$

components of the overall approach to using oligonucleotides as therapeutic agents. *Antisense* describes the interaction between oligonucleotides complementary to (sense) pre-mRNA[1] or mRNA molecules; these inhibit the oligonucleotides production of the protein product. The term has been broadened to describe any therapeutic oligonucleotide interaction with nucleic acids. *Triplex* denotes the interaction between oligonucleotides and double-stranded DNA that may result in inhibition of transcription. RNA structures display double-stranded regions, however, and thus the formation of triple-stranded structures in RNA is also possible. *Aptamers* describes the use of oligonucleotides to bind to nucleic acid binding proteins.

SCOPE AND OBJECTIVES OF THE REVIEW

This review focuses strictly on the use of oligonucleotides designed to interact with nucleic acids as therapeutics. The objectives are to place oligonucleotide therapeutics in the context of modern drug discovery and development and to summarize recent progress.

[1]Abbreviations: BFGF, basic fibroblast growth factor; cAMP, cyclic AMP; CH_3-P, methyl-phosphonate oligonucleotides; EGF, epidermal growth factor; ELAM, endothelial cell adhesion molecule; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; HB, hepatitis B; HIV, human immunodeficiency virus; HSV, herpes simplex virus; HTLV, human T-cell lymphotrophic virus; ICAM, intracellular adhesion molecule; IGF, insulin-like growth factor; IL-1, interleukin 1; introns, intervening sequences; IV, influenza virus; mRNA, messenger ribonucleic acid; P, phosphodiester oligonucleotides; P-acridine, phosphodiester oligonucleotide conjugated with acridine moiety; PCNA, proliferating cell nuclear antigen; PLA_2, phospholipase A_2; P-lipid, phosphodiester oligonucleotide conjugated with lipid moiety; PMA, phorbol mysteric acid; P-S, phosphorothioate oligonucleotides; Rev, regulation of virion protein expression; RSV, Rous sarcoma virus; TAR, Tat response element; TBE, tick-borne encephalitis; Tm, thermal transition point; VSV, vesicular stomatitis.

BASIC CONSIDERATIONS

Conceptually, oligonucleotide drug effects can be rationalized by traditional receptor theory and basic concepts concerning drug action. Within the broad context of pharmacological theory, however, a number of differences influence rational drug design and the potential utility of these agents.

Pharmacodynamics

RNA INTERMEDIARY METABOLISM Oligonucleotides are designed to modulate the information transfer from the gene to protein—in essence, to alter the intermediary metabolism of RNA. Figure 1 summarizes these processes.

RNA intermediary metabolism is initiated with transcription. The transcription initiation complex contains proteins that recognize specific DNA sequences and locally denature double-stranded DNA, thus allowing a member of the RNA polymerase family to transcribe one strand of the DNA (the antisense strand) into a sense pre-mRNA molecule. Usually during transcription, the 5' end of the pre-mRNA is capped by adding a methyl-guanosine and most often by methylation of one or two adjacent sugar residues. This enhances the stability of the pre-mRNA and may play a role in a number of key RNA processing events (2). Between the 5' cap and the site at which translation is initiated is usually a stretch of nucleotides; this area may play a key role in regulating mRNA half-life (3).

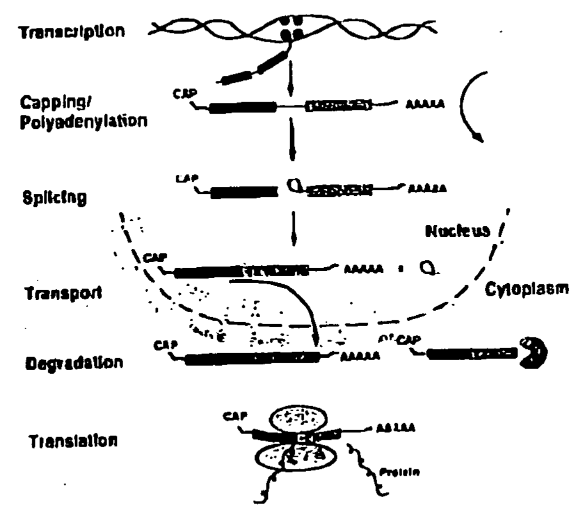


Figure 1 RNA processing.

Similarly, the 3' end of the pre-mRNA usually has a stretch of several hundred nucleotides beyond the translation termination signal. This area often plays an important role in determining mRNA half-life. Moreover, post transcriptionally, most pre-mRNA species are polyadenylated. Polyadenylation stabilizes the RNA, is important in transport of mature mRNA out of the nucleus, and may play important roles in the cytoplasm as well (4, 5).

Because eukaryotic genes usually contain intervening sequences (introns), most pre-mRNA species must have these sequences excised and the mature RNA spliced together. Splicing reactions are complex, highly regulated, and involve specific sequences, small molecular weight RNA species, and numerous proteins. Alternative splicing processes are often used to produce different mature mRNAs and, thus, different proteins. Even though introns are often considered waste, important sequences are conserved, and some introns may play a variety of regulatory roles.

Mature mRNA is exported to the cytoplasm and engages in translation. mRNA half-life varies from a few minutes to many hours and appears to be highly regulated (3).

Each step shown in the pathway is a composite of numerous steps, and each step is theoretically amenable to intervention with oligonucleotides. For virtually no mRNA is the pathway fully defined, however, and available information is insufficient to determine the rate-limiting steps in the intermediary metabolism of any mRNA species (6, 7).

AFFINITY The affinity of oligonucleotides for their receptor sequences results from hybridization interactions. The two major contributors to the free energy of binding are hydrogen bonding (usually Watson-Crick base pairing) and base stacking in the double helix that is formed. As mentioned, affinity is affected by ionic strength. Affinity results from hydrogen bonding between complementary base pairs; the reduction in entropy results from the stacking of the coplanar bases. Consequently, affinity increases as the length of the oligonucleotide receptor complex increases. Affinity also varies as a function of the sequence in the duplex. Nearest neighbor rules allow the prediction of the free energy of binding for DNA-DNA and RNA-RNA hybrids with relatively high precision (8, 9). Less information is available to develop predictions for DNA-RNA duplexes. A common misconception is that DNA-RNA duplexes are more stable than DNA-DNA duplexes. In fact, the relative stability of these duplexes varies as a function of the sequence. RNA-RNA duplexes are typically the most stable (S. M. Freier, unpublished results).

As with other drug-receptor interactions, activity requires a minimum level of affinity. For many targets and types of oligonucleotides, the minimum length of an oligonucleotide may be 12–14 nucleotides.

SPECIFICITY Specificity derives from the selectivity of Watson-Crick or other types of base pairing. The decrease in affinity associated with a mismatched base pair varies as a function of the specific mismatch, the position of the mismatch in a region of complementarity, and the sequence surrounding the mismatch. As an example, Table 2 compares the impact of various mismatches centered in two complementary 18-mers. The $\Delta\Delta G^0_{37}$ or change in Gibbs free energy of binding induced by a single mismatch varies from $^+0.2$ to $^+4.9$ kcal/mol per-modification at 100 mM NaCl. Thus, a single base mismatch results in a change in affinity of approximately 500-fold (10). Modifications of oligonucleotides may alter specificity. In fact, we have synthesized modified bases with substantially enhanced selectivity and others that display virtually no preferences for different bases.

Table 2 Effects of single-base mismatches on duplex stability. Absorbance vs temperature profiles were measured at 4 μM each strand in 100 mM Na^+, 10 mM phosphate, 0.1 mM, EDTA, pH 7.0.

X strand: 5'-d(CTC GTA CCx TTC CGG TCC)-3' fully phosphorothioate X = dA, dC, dG, or T

Y strand: 5'-(GGA CCG GAA yGG TAC GAG)-3' fully RNA y = A, C, G, or U

X	Y	T_m[a] (°C)	ΔT_m (°C)	ΔG^0_{37} (kcal/mol)	$\Delta\Delta G^0_{37}$ (kcal/mol)
dT	rA	53.6	—	−11.6	—
dT	rC	40.8	−12.8	−8.9	+2.6
dT	rG	50.0	−3.6	−10.5	+1.1
dT	rU	41.9	−11.7	−9.1	+2.5
dG	rC	56.9	—	−13.1	—
dG	rA	42.3	−14.6	−8.9	+4.2
dG	rG	45.0	−11.8	−9.3	+3.8
dG	rU	45.7	−11.1	−9.8	+3.3
dC	rG	59.0	—	−12.5	—
dC	rA	43.3	−15.7	−9.3	+3.2
dC	rC	39.5	−19.4	−8.7	+3.9
dC	rU	40.0	−19.0	−8.7	+3.8
dA	rU	52.7	—	−11.4	—
dA	rA	42.7	−10.0	−9.3	+2.2
dA	rC	42.7	−10.0	−9.1	+2.3
dA	rG	44.5	−8.1	−9.3	+2.1

[a] T_m and free energies of duplex formation were obtained from fits of the absorbance vs. temperature [illegible] to a two-state model with linear sloping baselines. Reported parameters are averages of at least three experiments.

Based on the differences in affinity of oligonucleotides for their complementary target sequence, calculations suggest that unmodified oligodeoxynucleotides between 11–15 in length should be able to bind selectively to a single RNA species in the cell (11). Studies in our laboratories have demonstrated that affinities predicted by nearest neighbor analyses are highly useful in rational drug design (10). For example, by using strategies based on nearest neighbor predictions, oligonucleotides can be designed that selectively inhibit the production of mutant RAS containing a single base change in the mRNA vs. normal RAS in cells in tissue culture (B. P. Monia et al, manuscript submitted).

NUCLEIC ACID SELECTIVITY The 2'-hydroxyl in RNA results in the sugar assuming a different conformation from that in DNA. RNA-RNA duplexes assume an A-form double helix whereas DNA-DNA duplexes assume a B-form double helix. Consequently, oligonucleotides can be modified to bind more tightly to RNA or DNA sequences. For example, Table 2 shows the effect of 2'-modifications at every position of a 15-mer on T_m and ΔG^0_{37} for DNA and RNA targets and demonstrates that 2'-O-methyl substitutions increase T_m for RNA by 1.4° per modification compared to DNA (12).

RNA STRUCTURE RNA can assume a variety of secondary structures deriving from intramolecular base pairing. The simplest structures are stem-loops in which double-stranded regions are interspersed with loops and random coils. More complex structures described as *pseudoknots* also form (13). These structures are profoundly important in determining RNA function and influencing the ability of oligonucleotides to bind to their RNA targets. The types of effects of bound oligonucleotides on RNA function are affected by RNA structures as well.

Pharmacokinetics

As with any other class of drugs, oligonucleotide drugs must attain a sufficient concentration at their receptor for a sufficient period to display activity. Inasmuch as most of the targets for oligonucleotides are intracellular, oligonucleotides must be relatively stable in and outside the cell and must be able to traverse the cellular membrane.

NUCLEASE STABILITY Oligonucleotides may be degraded by nucleases. Nucleases that degrade DNA or RNA from either the 5' or 3' terminus are known as *exonucleases;* those that cleave internally are *endonucleases*. Numerous nucleases exist and have been shown to degrade oligonucleotides. Although in serum the dominant nuclease activity is 3' exonuclease, in cells and other bodily fluids 3' and 5' exonucleases and endonucleases are present.

In serum, phosphodiester oligodeoxynucleotides are rapidly degraded. The rate of degradation varies as function of the sequence and length of the oligonucleotide and the type of serum (14–16). Typically, half-lives of phosphodiester oligodeoxynucleotides range from 15 to 60 min in most sera. Heat inactivation of serum reduces the rate of degradation of oligonucleotides. Oligoribonucleotides are significantly less stable than oligodeoxynucleotides.

Work from many laboratories has demonstrated that a wide range of modifications may be used to enhance the stability of oligonucleotides. Phosphate modifications have been shown to result in marked increases in stability (see Table 3). Phosphorothioate oligonucleotides have been shown to be extremely stable in media, cells and cell extracts, serum, various tissues, urine and stable to most nucleases (16–20). The half-life of phosphorothioate oligonucleotides is greater than 24 hr in nearly all environments tested. Furthermore, phosphorothioates have been shown to be stable to various restriction endonucleases when in duplexes. In general, one of the diastereomers is cleaved slowly and the other is entirely resistant (21–24).

The non-ionic methylphosphonate analogs have also been shown to be extremely stable to nucleases (25–31). Again, these oligonucleotides are diastereomeric at each modified phosphate, and the R isomer is slightly more sensitive than the S isomer to degradation by nucleases (16, 32).

Table 3 Effects of 2' modifications on hybridization and stability. Duplex hybridization was evaluated from absorbance vs. temperature profiles at 260 nm in 100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA, pH 7.0 at 8 μM strand concentration.

		Hybridization		Serum stability
Modification[1]	Positions	T_m[2] vs DNA (°C)	T_m vs RNA (°C)	$T_{1/2}$
Phosphodiester	All	50.5	39.0	1 h
Phosphorothioate	All	43.2		>24 h
2'-O-nonyl dA	12 13 14		41.3	60 h
2'-O-allyl	12 13 14	50.3	40.8	10 h
2'-O-benzyl	12 13 14	45.5	37.8	18 h
2'-O-aminopropyl	12 13 14	53.7	42.0	
				1 h
2'-fluoro P + S	2'F in 12 13 14 P = S in all	47.2	36.5	>>24 h

[1] 15 mer: CGA CTA TGC AAA AAC

[2] T_m is the temperature at which half the strands are in the duplex state and half are in the coil state. T_m was obtained from a nonlinear least squares fit of the experimental data to a modified two-state model with linear sloping baselines (224)

Other classes of modifications that have been reported to result in substantial nuclease stability include the phosphoramidates (19, 33) and isopropyl phosphate triesters (34, 35). Interestingly, ethylphosphate triesters were shown to be cleaved after being deethylated in cells (27, 36). Oligonucleotides containing α-anomers in the sugar moiety are substantially more stable in serum and cells than natural phosphodiesters (14–15, 37–41).

Modifications at the 2'-position of the sugar have also been shown to enhance nuclease stability (42). 2'-O-methyl-oligonucleotides were shown to be significantly more resistant than unmodified oligonucleotides, and 2'-O-allyl modified oligonucleotides were even more stable (43). In studies in our laboratory, a large number of 2'-modifications have been characterized. 2'-O-methyl analogs were highly resistant to nucleases in serum and cells. Modifications as bulky as nonyl groups were shown to have only a minor negative effect on hybridization and to impart high levels of nuclease stability In contrast, 2'-fluoro derivatives were nearly as sensitive to nucleases as unmodified oligoribonucleotides (12, 44). Table 3 provides a comparison of affinities to RNA and stabilities in serum for several 2' modified oligonucleotides (45). Although numerous other modifications have been studied, either insufficient data concerning hybridization properties or nuclease stabilities have been reported to support conclusions or their hybridization properties were unattractive. For example, open ring sugar analogs of adenosine were reported to be nuclease stable (46). Acyclic pentofuranosyl modified oligonucleotides were reported to be nuclease resistant, but the T_m for these oligonucleotides was reduced 9–15 degrees per modification (47). Other acyclic sugars have been reported but, again, the hybridization properties were poor (48). Carbocyclic modified oligothymidylates were reported to be nuclease resistant and to hybridize to oligodeoxyadenosine with higher affinity than natural oligodeoxy-thymidylate (49, 50), but studies on mixed sequences have not been reported.

A wide variety of phosphate replacements have also been studied. In earlier work, the phosphodiester was replaced with esters, amides, and various polymeric materials, but these modifications were not designed to be used as antisense oligonucleotides and, therefore, are largely unattractive (12, 16). More recently, formacetal replacement of the phosphate has been reported by two groups to result in oligonucleotides with acceptable hybridization properties and nuclease resistance (51–53).

Other modifications for which little information is available include sulfonamide replacement of phosphate (54), diphosphonate dinucleotides (55), acetamide linkages (56, 57), and phosphonyl methyl linkages (58). These and other modifications are discussed in detail in two recent reviews (12, 16). In our laboratories, a number of other novel backbone modifications have been synthesized. Given the number of novel synthetic approaches and molecules

and the number of laboratories now involved, a substantial increase in the repertoire of backbone-modified oligonucleotides with desirable properties is likely in the near future.

In addition to uniform modifications, a number of pendant groups at the 5' and/or 3' termini and more recently in internal positions of oligonucleotides have been reported to enhance nuclease stability. Modifications include intercalating agents (59–62) and poly-L-lysine (63, 64) at the 5' or 3' terminus and a number of modifications such as amino-alkoxy (65), anthraquinone (66), and alkyl groups (45). Moreover, heterocycle modifications, including pendant groups from the N2 site of guanine (67, 68), pendant groups from 3-deazaguanine (69), and 5- and 6-position modifications of deoxycytidine and thymidine (70), have shown increased stability to nucleases of varying levels.

In conclusion, numerous medicinal chemical strategies can be employed to create oligonucleotides with varying degrees of nuclease stability. The choice of the modification(s) employed is dictated by the level of stability desired and other desired properties of the oligonucleotides. It is now possible to design oligonucleotides that display excellent hybridization characteristics and half-lives, that range from minutes to several days when oligonucleotides are incubated with nucleases, serum, cells, or cell extracts.

INTRACELLULAR STABILITY Although considerable confusion and controversy exist with regard to the stability of oligonucleotides in cells in tissue culture and the ability to predict intracellular stability of oligonucleotides based on stability in sera, a consensus opinion is emerging. The nuclease activity of sera derived from different species varies. Fetal calf serum is more active than mouse serum, and human serum appears to have the least nuclease activity (G. D. Hoke, unpublished observations). All sera display substantial nuclease activity, however, and there are significant lot-to-lot variations. In all sera tested, 3' exonucleases constitute the primary nuclease activity (12, 16, 71). In a number of publications, fetal calf serum used in tissue culture experiments has been heated to inactivate nucleases. Again, however, conditions were not standardized, and in some lots of sera, heating to 65°C for 30 min does not inactivate all nucleases (16).

Another factor that has contributed to confusion is that a variety of labeling methods and analytical techniques have been employed. Studies have employed $3'^{32}P$ and $5'^{32}P$ labeled oligonucleotides, $5'^{35}S$ labeled oligonucleotides, and oligonucleotides labeled with fluorescent pendant groups at the 5' terminus (14–16, 72). Relatively few studies have used uniformly labeled oligonucleotides. Furthermore, relatively few studies have rigorously separated intact oligonucleotides from degradation products, and even fewer have performed careful kinetic studies.

Studies in our laboratory have employed either phosphodiester oligonucleotides uniformly labeled with ^{32}P or phosphorothioate oligonucleotides uniformly labeled with ^{35}S. The kinetics of degradation have been studied with several cell lines in vitro and cytoplasmic and nuclear extracts derived from HeLa cells. In contrast to a number of studies, in all cells studied to date, phosphodiester oligonucleotides were degraded within 15–30 min of incubation (71, 73). In contrast, phosphorothioate oligonucleotides of 15, 21, and 30 nucleotides in length and various sequences were stable for at least 24 hr when incubated with various cells. In studies in HeLa cells in which ISIS 1082, a 21-mer phosphorothioate, was incubated with the cells, then extracted from cells at various time points and analyzed on polyacrylamide gels, the compound was intact for four days (73).

Methylphosphonate oligonucleotides have also been shown to be stable in a variety of cells lines and extracts (14). No other class of oligonucleotides, however, has been sufficiently studied to allow definitive conclusions.

CELLULAR UPTAKE AND DISTRIBUTION Antisense oligonucleotides typically are 15–30 nucleotides long and thus have molecular weights that range from 4500–9000 daltons. The charge carried by phosphodiesters is, of course, negative and they are highly water soluble. The charge and hydrophilicity of modified oligonucleotides vary depending on the modifications. Consequently, membrane transport and cellular distribution are likely to vary widely as a function of the modifications introduced into oligonucleotides. For the two classes of modified oligonucleotides for which significant data have been reported—methylphosphonates and phosphorothioates—this is clearly the case. For both classes of oligonucleotides, the evidence is compelling that they do enter many cells at pharmacologically relevant concentrations.

Methylphosphonates are uncharged and lipophilic. Although thought to be taken up by most cells in tissue culture via passive diffusion, detailed studies of the kinetics of cellular uptake, distribution, and metabolism of uniformly labeled methylphosphonates have not been reported. Studies in Syrian hamster fibroblasts on oligonucleotides 3–9 nucleotides in length showed linear cell association for 1 hr, then reduced uptake. At equilibrium, the intracellular concentration of oligonucleotide was reported to be equivalent to the extracellular concentration (27, 74). In another study, a 21-mer methylphosphonate labeled with ^{32}P at the 5' terminus was reported to be taken up by CV-1 cells. Cell association was linear for 2 hr. Unfortunately, however, studies proving that the cell-associated radioactivity represented intact oligonucleotide were not presented. Nor were detailed studies on characteristics of uptake or intracellular distribution presented (75).

Phosphorothioates are negatively charged, but because of the sulfur atoms

they may be slightly more lipophilic than phosphodiesters and tend to bind nonspecifically to serum proteins. Studies in our laboratories have shown that phosphorothioate oligonucleotides bind to serum albumin and that in the presence of serum albumin, cell-association is reduced (73; G. D. Hoke et al, unpublished observations).

Studies employing a 28-mer phosphorothioate deoxycytidine that was uniformly labeled with ^{35}S demonstrated that when HeLa cells were incubated with 1 μM of the drug, significant intracellular concentrations were achieved. Cellular uptake was linear, reaching a plateau of 60 p mole/10^6 cells in 6 hr. Adsorption to the cell membrane was minimal. Uptake was also concentration-dependent, reaching a plateau at approximately 1 μM. The drug associated with HeLa cells was intact for 24 hr and was located in both nuclei and cytoplasm. Infection with herpes simplex virus type 2, but not type 1, increased cellular uptake (76).

Studies in our laboratories have confirmed and extended the observations on phosphorothioate oligonucleotides. The cellular uptake, distribution, and metabolism of ISIS 1082, a uniformly ^{35}S labeled 21-mer phosphorothioate with a mixed antisense sequence, have been characterized in HeLa cells and HeLa S_3 cells, a variant line conditioned to growth in suspension. Incubation of HeLa cells with 5 μM of the drug resulted in approximately 8% of input radioactivity being associated with the cells. Cell association was linear for approximately 8 hr, and approximately 20% of the cell-associated radioactivity appeared to be adsorbed to the membrane. Uptake was temperature-dependent, required viable cells, and was inhibited by metabolic poisons. Uptake was concentration-dependent, and was linear to 10 μM. Uptake was influenced slightly by calcium and magnesium and was saturable. Natural oligonucleotides and methylphosphonates did not compete for uptake while other phosphorothioates competed; however, different length and sequence phosphorotioates competed differently (73, 77).

We have also studied other phosphorothioates of various lengths and other cell lines. HL 60 cells appear to take up less phosphorothiate oligonucleotides than HeLa cells and HeLa S_3 cells take up very little drug (73). Although not directly compared, human umbilical vein endothelial cells also appear to take up less drug than HeLa cells. Thus, there is considerable variation in the extent of uptake as a function of cell type.

In all cells studied, and with all uniformly labeled phosphorothioate oligonucleotides of varying size and sequences, we have shown that these drugs are stable in cells and cytoplasmic and nuclear extracts. In HeLa cells, no degradation of intracellular ISIS 1082 was observed for four days (73). Preliminary studies confirmed that these oligonucleotides distributed to both cytoplasm and nuclei and showed that there is an active temperature-dependent efflux process as well (77, 78).

When incubated with cells in the absence of serum or heat-inactivated serum, several laboratories have reported the apparent uptake of phosphodiester deoxyoligonucleotides. Moreover, a number of laboratories have reported activities for phosphodiester oligonucleotides that apparently were due to cellular uptake and intracellular activities. The studies on cellular uptake are not fully convincing, however Loke et al (79) studied deoxythymidine oligonucleotides ranging from 3–20 nucleotides in length and labeled with acridine at the 3' terminus. They incubated HL60 and three other hematopoietic cell lines with 12.5 μM of the acridine labeled drug and used flow-cytometric analyses of acridine fluorescence to quantitate cellular uptake. Uptake was reported to decrease as the length of the oligonucleotide increased and to vary as a function of the cell type. Uptake achieved a plateau in HL60 cells in 50 hr and was inhibited by polynucleotides of any length. The authors concluded that the oligonucleotides were taken up by an endocytotic mechanism. Unfortunately, the stability of the oligonucleotide-acridine conjugate was not rigorously documented. Nor were possible effects of acridine in the uptake of the oligonucleotide rigorously explored. Additionally, possible quenching or enhancement of the fluorescence of acridine by cellular interactions was not explored. Finally, extrapolations from homopolymers to mixed sequences have not been proven to be valid.

Another study employing phosphodiester oligonucleotides reached similar conclusions (80). Again, for most of the experiments, oligodeoxythymidines of 8 to 16 nucleotides in length were incubated with L929 mouse fibroblasts in the absence of serum. Maximal uptake occurred within 2 hr and upon incubation with fresh medium, cell-associated ^{32}P was released. Substantial degradation of the 5' labeled oligonucleotide was observed within 2 hr, and the authors concluded that approximately 20% of the radioactivity was in nuclei. Again, the authors concluded that the most likely mechanism of uptake was endocytosis (80).

Other pendant modifications of phosphodiester oligonucleotides have also been studied. A 9-mer labeled with acridine at the 3' terminus was reported to be taken up by *Trypanosome brucei* (61). More recently, the same group has reported that a 9-mer coupled at the 3' terminus to acridine via a dodecanal linker was more active in cells expressing mutated RAS than a 9-mer with a 3' acridine only (81). 3' poly-L-lysine-oligonucleotides have been reported to be stable to serum nucleases and to have enhanced activity as compared to phosphodiesters. Uptake was not studied, however, (63, 82, 83). In a later publication, the uptake of a poly-L-lysine oligonucleotide conjugate was enhanced compared to the unmodified oligonucleotide (84). When used to treat cells other than L929 cells, however, poly-L-lysine conjugates were inactive (64).

A number of lipid conjugates have also been studied. 5' linked triethylammonium 1,2 di-O-hexadicyl-rac-glycerol-3-H-phosphonate oligonucleotides were taken up 8–10-fold more than unmodified oligonucleotides by L929 cells and were more active against varicella zoster viral infections, albeit at high concentrations (85). An oligonucleotide linked at the 5' terminus to an undecyl residue was reported to be active, but no uptake or stability studies were reported (86).

The intracellular fate of oligonucleotides injected into oocytes and the uptake of oligonucleotides into oocytes have also been studied. When injected into *Xenopus* oocytes, unmodified oligonucleotides were degraded within 1 min primarily by 3' exonuclease digestion, but other nucleolytic activities were also present (87, 88). Interestingly, in this system, even phosphorothioate oligonucleotides were reported to be degraded, albeit much more slowly than phosphodiesters (89). These observations were extended in studies on oligodeoxynucleotides injected into CV-1 endothelial cells. A 28-mer oligonucleotide of either phosphodiester, phosphorothioate, or methylphosphonate type was injected into the cytoplasm of these cells. All three types of oligonucleotides localized to the nucleus in a temperature- but not energy-dependent fashion. The methylphosphonate oligonucleotide concentrated in regions of genomic DNA, in contrast to the two other oligonucleotides that co-localized with small nuclear ribonucleoproteins (90). Uptake of unmodified oligonucleotides by pre-implantation embryos was reported to be virtually nil (91).

Liposomes and related formulations have been shown to enhance cellular uptake of oligonucleotides in vitro. Loke et al (92) compared the uptake of phosphodiester and phosphorothioate deoxythymydine heptamers into HL-60 cells by using oligonucleotides coupled to 2-methyloxy-6-chloro 9-(5-hydroxypentyl) amino acridine and monitoring with flow cytometry. They did not determine the integrity of the oligonucleotides, but reached the conclusion that phosphodiester dT_7 was taken up by HL-60 cells much more effectively than phosphorothioate d-T_7, and that uptake plateaued at 50 hr. They reported increased anti-*c-myc* activity of phosphorothioate oligonucleotides after loading them in phosphatidyl serine liposomes. The uptake of a tetramer 2'-5' deoxyadenylate into L1210 cells was reported to be increased by loading the oligo-adenylate into *Staphyloccus aureus* protein A-crosslinked phospholipid vesicles (93). In our laboratories, we have shown that lipofectin, a cationic lipid mixture, can significantly increase the uptake and activity of phosphorothioate oligonucleotides in several cell lines. It also alters the intracellular distribution of these nucleotides (78).

With the exception of methylphosphonates, the conclusion from studies that have addressed the mechanisms of uptake of oligonucleotides is that the most likely mechanism is receptor-mediated endocytosis. In fact, in one study an 80-kd protein that appeared to bind oligonucleotides was partially purified

and postulated to be a "receptor" (79). The evidence supporting this mechanism is limited, however, and data are insufficient to conclude that receptor-mediated endocytosis is the most common or only mechanism of uptake of charged oligonucleotides in most cells.

In conclusion, although many questions remain to be answered, it appears that many cells in tissue culture may take up oligonucleotides at pharmacologically relevant concentrations. Clearly, oligonucleotides of different types behave differently and there are substantial variations as a function of cell type. Moreover, length and specific sequences may alter uptake, and pendant modifications may profoundly influence cellular uptake.

Once in the cell, it would seem that oligonucleotides distribute to the cytoplasm and the nuclei. In most if not all cells, phosphodiester oligonucleotides are rapidly degraded whereas methylphosphonates and phosphorothioates are much more stable. Again, pendant modifications may alter the rate of intracellular degradation and distribution.

Mechanisms of uptake and distribution are poorly understood. Clearly, however, multiple mechanisms may play a role, and different types of oligonucleotides may behave very differently.

Novel formulations may enhance cellular uptake. Liposomes and cationic lipids significantly enhance uptake and may alter the mechanisms of uptake and intracellular fate of oligonucleotides.

IN VIVO PHARMACOKINETICS Preliminary in vivo pharmacokinetic data are now available on methylphosphonate and phosphorothioate oligonucleotides. A 12-mer ^{3}H-labeled methylphosphonate injected in the tail vein of mice was rapidly cleared as intact oligonucleotide and distributed broadly to all tissues except the brain (94).

More extensive studies have been performed on ^{35}S-labeled phosphorothioates in rats. A true distribution phase of 15–25 min was observed after a single IV dose of a 27-mer followed by a prolonged elimination phase of 20–40 hr (94). The prolonged elimination phase may result from the binding of phosphorothioates to serum proteins. Phosphorothioates distrubuted broadly to all tissues except the brain and were eliminated in the urine intact. Phosphorothioates were rapidly and extensively absorbed after IM and IP administration (94).

Repeated daily doses of 50 mg/kg of a 27-mer phosphorothioate to mice resulted in similar distribution and elimination kinetics but slight differences in tissue concentrations from single dose studies. Liver, kidney, spleen, and lung were the organs with highest concentrations. Again, the drug was excreted intact in the urine (94).

Continuous osmotic pump administration of the same compound subcutaneously for 4 wk at doses of 50–150 mg resulted in similar pharmacokinetics (94).

Studies with ISIS 1082, a 21-mer phosphorothioate, in mice showed that when applied to the cornea in a sodium acetate buffer, significant adsorption to the cornea and absorption into the aqueous and vitreous humors occurred. Moreover, significant systemic bioavailability was observed (78). In rabbit, as much as 25% of an applied ocular dose was systemically bioavailable (unpublished observations). Post absorption pharmacokinetics were equivalent to IV pharmacokinetics.

Recently, a 20-mer phosphodiester was administered intravenously to rabbits. Clearance from blood was rapid and, after 90 min, 16% of the dose was found in the urine and was intact. In blood, at least 17% of the drug was estimated to be completely degraded within 5 min (95).

Toxicology

IN VITRO

Phosphodiesters Very little information has been published on the in vitro toxicities of unmodified oligonucleotides. In most systems, the oligonucleotides are thought to be rapidly degraded. When a 15-mer complementary to a c-myc sequence was incubated with human lymphocytes at 30 μM for 4 hr, no toxicity was observed. Longer incubation (24 hr) in 10% serum resulted in reduced ^{3}H-thymidine incorporation, but the authors concluded that this was probably due to dilution of the thymidine pool by thymidine liberated after rapid degradation of the oligonucleotide (96).

The incubation of a transformed leukemic cell line with 50 μM of a 20-mer complementary to a sequence in the BCL-1 proto-oncogene was reported to result in no decrease in viability as judged by trypan blue exclusion (97).

Methylphosphonates Incubation of Vero cells with 30 μM and lower concentrations of an 8-mer methylphosphonate for 24 hr resulted in no decrease in growth rate or cell count; however, 48 hr incubation resulted in 40% inhibition of growth rate (98). Similarly, neither of three 9-mers had any effect on L929 cell plating efficiency or protein synthesis after 16 or 40 hr incubations with 150 μM of drug (99). Incubation of T15 cells with 80 μM of a 9-mer directed against N-ras for 48 hr produced no effect on protein synthesis or viability (100). Similar results were reported for HT29 cells.

Inasmuch as methylphosphonate oligonucleotides have, when they have displayed activity, effective concentrations of 50–100 μM, the therapeutic index in vitro may be rather modest. Much more detailed studies are required before reaching final conclusions, however.

Phosphorothioates Phosphorothioate oligonucleotides bind to a variety of proteins, including serum albumin. In cell free protein translation experiments, they have been shown to induce nonspecific inhibition of protein synthesis (11, 101, 102). In wheat germ and rabbit reticulocyte lysate assays,

concentrations as high as 100 nM of a 17-mer phosphorothioate targeted to the protein mRNA inhibited globin synthesis relatively specifically. At 10 μM, nonspecific effects were observed (103). The nonspecific effects of phosphorothioates in these assays are length-dependent, as a 5-mer was much less potent than the 14-mers and dC28 appeared to be the most potent phosphorothioate oligonucleotide tested. In studies in our laboratories, we have made similar observations with a number of phosphorothioate oligonucleotides (G. D. Hoke et al, unpublished observations).

Phosphorothioate oligonucleotides have also been shown to inhibit DNA polymerases, reverse transcriptases, and nucleases when incubated in cell free systems (76, 77, 104).

Despite the potential nonspecific interactions of phosphorothioate oligonucleotides with cellular proteins, a wide variety of compounds have been shown to have excellent therapeutic indices. Microinjection of nanomolar concentrations of a 17-mer into *Xenopus* oocytes inhibited β-globin synthesis. When 16 μM of the compound were injected, however, protein synthesis was aborted and the oocytes underwent extensive cytolysis (89).

Incubation of cells in vitro with phosphorothioate oligonucleotides has likewise resulted in toxicities only at concentrations much higher than those at which therapeutic activities were observed. Human mononuclear cells were unaffected after 20 hr of incubation with 25 μM of several 15-mers (105). T697 cells were unaffected by a three-day exposure to 25 μM of a 20-mer (97, 106).

In our laboratories, we have determined the effects of ISIS 1082, a 21-mer phosphorothioate that inhibits herpes simplex virus types 1 and 2 infections in HeLa cells at 200–400 nM, on HeLa cell viability, DNA synthesis, RNA synthesis, protein synthesis, and energy metabolism. At no concentration below 500 μM were statistically significant effects observed after incubation for 96 hr. Exposure of HeLa cells to 500 μM ISIS for 48 hr resulted in 20% inhibition of protein synthesis (77). Similar results were observed in other cell lines.

Table 4 presents results from studies on 20 phosphodiester or phosphorothioate oligonucleotides targeted to various regions in the 5-lipoxygenase gene. Again, most of the phosphorothioates displayed toxicities only at 50 μM and greater. The exceptions to this rule were three 30-mers that inhibited cell growth at 10–35 μM. Clearly, one can conclude from this study that toxicity was time- and concentration-dependent and that, with longer exposures in particular, phosphorothioates were more toxic than their phosphodiester analogs (73).

We have identified other factors that influence the toxicity of phosphorothioates. Cell type may alter toxicity significantly. A comparison of the toxic effects of a 15-mer phosphorothioate on HL60 cells, U937 cells, and RBL-1

Table 4 In vitro toxicities of 5-lipoxygenase oligonucleotides in HL-60 cells[a]

Compound	Oligonucleotide Class[b]	Length	Sequence	AT:GC	24 hr	48 hr	72 hr	96 hr
1787	PD	15	5'GTGTGCCACCAGCAG-3'	1:2	21.5	18.5	16.0	14.4
1788	PS	15			>100	35.0	25.0	19.0
1789	PD	30	AATGGTGAATCTCAC		>100	>100	>100	>100
1790	PS	30	GTGTGCCACCAGCAG	1:1.1	>100	>100	15.0	11.8
1795	PD	15	TGCCAGTGATTCATG	1:0.88	63.0	39.5	34.0	26.0
1796	PS	15			>100	>100	50.0	35.0
1797	PD	30	OOTCTTCCTGCCAGT		>100	>100	50.0	74.0
1789	PS	30	GATKATGACCCGCT	1:1.31	>100	20.0	10.0	10.0
1799	PD	15	GTCCTGATGGCTTCC	1:1.5	28.0	25.0	22.0	22.0
1800	PS	15			>50.0	50.0	50.0	34.0
1801	PD	30	GTCCTGATGGCTTCC		>50.0	>50.0	>50.0	>50.0
1802	PS	30	CACACCAGGAGCCCG	1:2.0	35.0	27.0	21.0	3.9
1812	PD	15	GTTGCTCCTTGGTGT	1:1.14	29.0	17.0	16.0	18.0
1813	PS	15			>50.0	32.0	25.0	43.0
1814	PD	30	ATTTGCTGTTGCTGC		>50.0	32.0	25.0	
1815	PS	30	TTGGTGTGGAAATGC	1:0.88	10.0	9.0	10.0	43.8
1816	PD	15	AGGTGTCCGCATCTA	1:1.14	150.0	250.0	>50.0	>50.0
1817	PS	15			32.0	>50.0	>50.0	>50.0
1818	PD	30	TCGGCGCGCGGTCC	1:2.33	>50.0	>50.0	>50.0	>50.0
1819	PS	30	AGGTGTCCGCATCTA		15.0	19.0	19.0	20.0

[a] HL 60 cells were incubated in 96 well plates with increasing concentrations of oligonucleotides (1-50 or 100 μM) in the presence of 10% fetal bovine serum. Viability of the cells was determined at each time point by trypan blue exclusion. IC_{50} values were obtained by plotting percentage control cell number vs. drug concentration.

[b] PD = Phosphodiester; PS = Phosphorothioate.

cells showed considerable variation in sensitivity; HL60 cells were the most sensitive. As phosphorothioates bind to serum albumin, in the presence of 10% fetal calf serum, a 15-mer produced no cytotoxicity after 24 hr of incubation at 100 μM. In the presence of 2.5% fetal calf serum, the IC_{50} was 19 μM. Finally, the purity of the oligonucleotide has a significant effect. Purification of oligonucleotides in triethyl ammonium buffers with trityl-on HPLC followed by removal of the trityl groups in triethyl ammonium may result in substantial contamination with triethyl ammonium ions, which are toxic to cells (73). Others have alluded to batch-to-batch variations and the potential that contaminants might contribute to toxicities, but they have not identified potential toxins (60, 97, 106–108).

Pendant group modified oligonucleotides Limited information is available concerning the effects of pendant groups on the toxicities of oligonucleotides. An acridine conjugated 7-mer phosphodiester was reported to produce no toxicities at 100 μM even though the free acridine had an IC_{50} for cell viability of 2 μM (109). Two 11-mer phosphorodiesters that were covalently attached to an undecyl group at the 5' terminus had no apparent toxic effect on MDCK cells at 100 μM (86). 5'terminal phospholipid conjugates of both phosphodiester and phosphorothioate oligonucleotides produced little toxicity in L292 cells when incubated at 50 to 100 μM (85). In contrast, a phosphodiester 15 mer linked to poly-L-lysine was toxic to L929 cells at 1 μM (84).

Table 5 summarizes published data concerning the in vitro toxicology.

[illegible] Although only preliminary toxicologic data are available, considerably more information should soon be available, as several compounds are currently in preclinical development.

Single-dose toxicity studies in mice were reported for phosphodiester (19), methylphosphonate (110), phosphomorpholidate, and phosphorothioate oligonucleotides. Unmodified oligonucleotides resulted in deaths in two of four treated mice at 160 mg/kg and all four mice treated with 640 mg/kg IV. Within three days after injection, a phosphorothioate oligonucleotide resulted in equivalent toxicities to the phosphodiester. The other analogs produced similar toxicologic effects with slight differences in doses.

Single doses of as much as 3.5 mg of a 27-mer complementary to the REV gene of HIV given IV or IP produce no toxicities in rats. Daily injections of 50 mg/kg IV of the same compound for 12 days in mice resulted in no observable toxicities. This 27-mer was also administered via a subcutaneous osmotic pump designed to administer up to 150 mg at a constant rate for 4 wk to rats. Again, no toxicities in any organ were observed (94).

ISIS 1082, a 21-mer phosphorothioate targeted to inhibit herpes virus types 1 and 2, has been administered topically to mouse and rabbit eyes for as much as 21 days and resulted in no ocular toxicities. In rabbits, other organs were examined, and no effects were observed. Given the extensive bioavilability of ISIS 1082 in rabbits after ocular administration, this constitutes a significant observation.

Single doses of ISIS 2105, a 20-mer phosphorothioate active against human papilloma viruses, were administered intradermally and resulted in no local or systemic toxicities.

Consequently, a growing body of data supports the contention that at least single doses of phosphorothioate oligonucleotides may be given to mice, rats, and rabbits without significant acute or subacute toxicities.

MUTAGENICITY Virtually no data have been published on the potential mutagenicity of oligonucleotides. A 27-mer phosphorothioate was reported to be negative in an Ames assay in the presence or absence of a liver metabolic activation system at doses as high as 5 mg/plate (101).

P. Iverson (personal communication; 101) compared a number of oligonucleotide types and related chemicals in hamster lung fibroblasts. Unfortunately, although this study has been cited, the primary data have never been published, and thus it is difficult to draw any conclusion.

Mechanisms of Action of Oligonucleotides Interacting with Nucleic Acid Targets

The mechanisms by which interactions of oligonucleotides with nucleic acids may induce biological effects are complex and potentially numerous. Furthermore, very little is currently understood about the roles of various mechanisms or the factors that may determine which mechanisms are involved after oligonucleotides bind to their receptor sequences. Consequently, a discussion of mechanisms remains largely theoretical. Although a number of potential schemes to classify mechanisms of action might be employed, I prefer a scheme based on drug-receptor concepts.

OCCUPANCY-ONLY MEDIATED MECHANISMS Classic competitive antagonists are thought to alter biological activities because they bind to receptors, thereby preventing natural agonists from binding and inducing normal biological processes. Binding of oligonucleotides to specific sequences may inhibit the interaction of the RNA or DNA with proteins, other nucleic acids, or other factors required for essential steps in the intermediary metabolism of the RNA or its utilization by the cell.

Transcriptional arrest Oligonucleotides may bind to DNA and prevent either initiation or elongation of transcription by preventing effective binding of factors required for transcription, thus producing transcriptional arrest.

Table 5 In Vitro toxicology of antisense oligonucleotides

Oligonucleotide class	Length	Concentration	Target	Cell type	Time	Toxicity assessment	References
Phosphodiester	15	30 µM	c-*myc*	Human T cells	4 hours	Nontoxic	99
Phosphodiester	20	150 µM	βCL2	697 cells	3 days	Nontoxic	100
Phosphorothioate	20	25 µM	βCL2	697 cells	3 days	Nontoxic	
Phosphodiester	23	1-30 ng	Vg 1	*Xenopus* oocytes	2 days	Nontoxic @ low concentrations, i.e. <5 ng	92
Phosphorothioate	23	1–30 ng	Vg1	*Xenopus* oocytes	2 days	Toxic @ 15-30 ng	
Phosphodiester-acridine conjugate	7	50-100 µM	Type A influenza	MDCK	3 days	Nontoxic	112
Acridine alone		2 µM		MDCK	3 days	Toxic	
Methylphosphonate	9	150 µM	VSV	L929	16,40 hours	Nontoxic	102
Methylphosphonate	9	80 µM	N-i-OS	T15	48 hours	Nontoxic	103
Methylphosphonate	9	80 µM	N-*ras*	HT29	48 hours	Nontoxic	
Phosphodiester	14	1-25 µM	HIV	ATH8	1 days	Minor toxicity @ all conc. (<35%)	20
Methylphosphonate	14	1-25 µM			7 days	Minor toxicity @ all conc. (<27%)	
Phosphorothioate (heteropolymer)	14	1-25 µM			7 days	Nontoxic	
Phosphorothioate (homopolymer)	14-28	1-25 µM			7 days	Nontoxic	76
Phosphorothioate (homooligomer)	28	3-50µM	HSV-2	HeLa S_3	72 hours	Nontoxic	
Phosphorothioate (antisense)	20	4-100 µg/ml	HIV	H9, MOLT3	96 hours	Nontoxic	19
Phosphorothioate (nonsense)	20	4-100 µg/ml		H9, MOLT3	96 hours	Nontoxic (4 µg/ml) Toxic (20 µg–5% 100 µg-67%)	
Phosphodiester homooligomer (dT)	15	4-100 µg/ml		H9, MOLT3	96 hours	Toxic	
Phosphodiester homooligomer (dA)	15	4-100 µg/ml		H9, MOLT3	96 hours	Minor toxicity	
Phosphormorpholidate homooligomer (dT)	15	4-100 µg/ml		H9, MOLT3	96 hours	Toxic	
Phosphormorpholidate homooligomer (dG)	15	4-100 µg/ml		H9, MOLT3	96 hours	Toxic	
Phosphorothioate	17	16 µM	β-Globin	*Xenopus* oocytes	>6 hours	Toxic	106
Phosphorothioate	15	0.1–25 µM	ILIβ	Human blood monocytes	20 hours	Nontoxic	108

It is possible that oligonucleotides could bind to segments of DNA that are partially denatured by the transcription complex, although this is highly unlikely. The initiation and elongation of transcription require a complex set of proteins and other factors, and it is difficult to conceive of a mechanism by which oligonucleotides might compete effectively against the transcriptional machinery for these single-stranded regions. Nevertheless, despite the improbability of such an event, reports of activities have been made that can be explained most simply by this mechanism (112, 113). Additionally, Helene and colleagues (114) reported that hexanucleotides to nonanucleotides with acridine derivatives at the 3' terminus inhibited transcription of the β-lactamase gene. When the RNA polymerase was preincubated with the oligonucleotide-acridine adducts, however, they observed nonspecific inhibition (115).

The alternative to seeking transient single-stranded regions or to attempting to denature a double-stranded region of DNA is to inhibit transcription by interacting with double-stranded DNA, i.e. forming triple-stranded structures. To form triple-stranded structures, hydrogen bonds other than Watson-Crick must be formed. In most current triple-strand motifs, the oligonucleotide becomes the third strand by recognizing hydrogen bonding donor/receptor sites on a purine reference strand and lying in the major groove (116–124). Alternative motifs have also been proposed. For example, Hogan and colleagues (125) proposed that a purine-rich oligonucleotide can form a triplex structure based upon the purines in the oligonucleotide base pairing in parallel fashion with the purines in the duplex DNA. Studies by Dervan's group (126), however, suggested that the purine-rich oligonucleotide bound to the duplex DNA with an antiparallel orientation.

The formation of triple-stranded structures by using natural nucleosides requires runs of purines Watson-Crick-hydrogen-bonded to their complementary pyrimidines. When cytidine is used to form a triple strand with a G-C base pair, it must be protonated; this occurs at nonphysiological acidic conditions (121). Furthermore, all motifs employ one or more "weak" hydrogen bonds. Thus, to achieve sufficient stability, relatively long triple-strand structures are required.

The principal theoretical advantage of triple helical inhibition schemes is that transcription represents the first step in the intermediary metabolism of RNA and may, therefore, provide substantial leverage for drug therapy. The other advantages that have been suggested are much more speculative. For example, it has been suggested that the smaller number of genes (one or two) compared to the number of mRNA molecules (usually less than 1000) per cell is an advantage for approaches that inhibit transcription. This suggestion ignores the kinetics of the targets, however. Genes have an infinite half-life relative to cell life. RNA molecules are synthesized and degraded with

varying kinetics. Furthermore, a variety of mechanisms exist to assure that even covalent modifications of DNA are repaired. Another concept has been that triple helixes in DNA might produce permanent biological effects. That even alkylating and DNA-cleaving anticancer drugs do not produce permanent effects points to the speciousness of this notion.

A number of theoretical disadvantages of triple helical inhibition of transcription have also been enumerated. Sequence specific binding is not yet possible, as runs of homopyrimidines are required. These sequences may play important regulatory roles in DNA, as they are much more abundant than statistically predicted (16). Longer term, a more substantial problem may simply be gaining sequence-specific access to DNA in chromatin. Additionally, deliberate interactions with the genome raise concerns about mutagenicity, carcinogenicity, and teratogenicity, which, in most therapeutic settings, are of considerable importance.

Several strategies have been developed to circumvent the requirement for purine-pyrimidine runs and other limitations. For example, purine oligonucleotides form triplex structures at higher pH values than pyrimidine-rich oligonucleotides (125, 126). Similarly, pyrimidine-rich oligonucleotides, in which 2'-O-methyl pseudoisocytidine was substituted for 2' deoxycytidine, formed triplex structures as neutral pH (127). Oligonucleotides with linkers that allow crossover of the oligopyrimidine from one strand of the duplex to the other have been reported and this motif suggested to be a solution to a broader sequence repertoire (128). To enhance the stability of triple helices, intercalators and photoactivatable crosslinkers and alkylators have been conjugated to oligo pyrimidines (129–131). To increase potency and enable identification of sites of binding, a number of cleavage moieties have been conjugated to oligopyrimidines (132–137). Finally, to enhance nuclease stability, methylphosphonates (138) and α-oligonucleotides (136) have been shown or suggested to form triple helices.

In addition to cleavage of DNA in vitro by triplex-forming oligonucleotides coupled to cleavage reagents and alkylation induced by oligonucleotide-coupled alkylators, several other methods have been used to show triplex formation. These include agarose affinity column purification (139), NMR (140), protection from uv dimerization (141), solution hybridization (142), inhibition of binding of DNA-binding proteins (143), inhibition of restriction endonucleases (144), and repression of c-myc transcription in vitro (125). Recently, a 28-mer phosphodiester stabilized at the 3' end by alanine and directed to enhancer elements for the IL-2 receptor gene was shown to inhibit the transcription of the gene when incubated with human lymphocytes. The authors reported evidence for selectivity to oligonucleotides as well (145).

Obviously, triple-helix-based inhibition of transcription is of potential therapeutic importance, particularly for targets that for a variety of reasons

may be difficult to inhibit at the post-transcriptional level. Substantial medicinal chemistry must be completed, however, to create oligonucleotides that can interact with duplex structures in a sequence-specific fashion without requiring special motifs. Once this is accomplished, of course, additional studies must show that the other theoretical limitations discussed above can be overcome.

Inhibition of splicing A key step in the intermediary metabolism of most mRNA molecules is the excision of introns. These "splicing" reactions are sequence-specific and require the concerted action of spliceosmes. Consequently, oligonucleotides that bind to sequences required for splicing may prevent binding of necessary factors or physically prevent the required cleavage reactions. This then would result in inhibition of the production of the mature mRNA. Although there are several examples of oligonucleotides directed to splice junctions, none of the studies presented data showing inhibition of RNA processing, accumulation of splicing intermediates, or a reduction in mature mRNA. Nor are there published data in which the structure of the RNA at the splice junction was probed and the oligonucleotides demonstrated to hybridize to the sequences for which they were designed (146–149). Activities have been reported for anti-c-*myc* and antiviral oligonucleotides with phosphodiesters, methylphosphonates, and phosphorothioates.

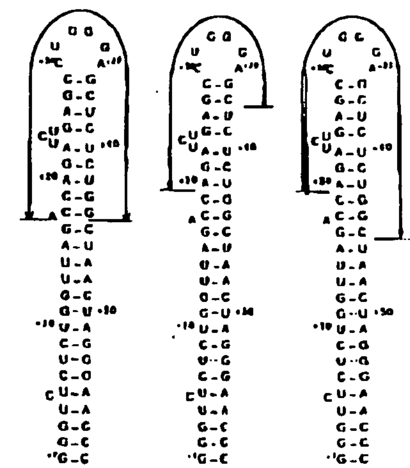
Translational arrest Without question, the mechanism for which the majority of oligonucleotides have been designed is translational arrest. Oligonucleotides have been designed to bind to the translational initiation codon. The positioning of the initiation codon within the area of complementarity of the oligonucleotide and the length of the oligonucleotide used have varied considerably. Again, unfortunately, only in relatively few studies have the oligonucleotides been shown to bind to the sites for which they were designed, and other data that support translation arrest as the mechanism reported.

Target RNA species that have been reported to be inhibited include HIV (19), vesicular stomatitus virus (VSV) (82), n-*myc* (150), and a number of normal cellular genes (151–154).

In our laboratories, we have shown that a significant number of targets may be inhibited by binding to translation initiation codons. For example, ISIS 1082 hybridizes to the AUG codon for the UL13 gene of herpes virus types 1 and 2. Studies with RNaseH confirmed that ISIS 1082 binds selectively in this area. In vitro protein synthesis studies confirmed that ISIS 1082 inhibited the synthesis of the UL13 protein, and studies in HeLa cells showed that it inhibited the growth of herpes type 1 and type 2 with an IC_{50} of 200–400 nm by translation arrest (155). Similarly, ISIS 1753, a 30-mer phosphorothioate complementary to the translation initiation codon and surrounding sequences of the E2 gene of bovine papilloma virus, was highly effective, and its activity was shown to be due to translation arrest. ISIS 2105, a 20-mer phosphorothioate complementary to the same region in human papilloma virus, was shown to be a very potent inhibitor. Compounds complementary to the translation initiation codon were the most potent of the more than 50 compounds studied complementary to various other regions in the RNA (156).

In conclusion, translation arrest represents an important mechanism of action for antisense drugs. A number of examples purporting to employ this mechanism have been reported. Recent studies on several compounds have provided data that unambiguously demonstrate that this mechanism can result in potent antisense drugs.

Disruption of necessary RNA structure RNA adopts a variety of three-dimensional structures induced by intramolecular hybridization, the most common of which is the stem loop (Figure 2). These structures play crucial roles in a variety of functions. They are used to provide additional stability for



length	28	18	26
P=S	1972	1308	1307
P=O	1971	1116	1118

Figure 2 Antisense oligonucleotides directed against the HIV TAR element. The oligonucleotide sequences are complementary to the tar sequences where indicated.

RNA and as recognition motifs for a number of proteins, nucleic acids, and ribonucleoproteins that participate in the intermediary metabolism and activities of RNA species. Thus, given the potential general activity of the mechanism, it is surprising that occupancy-based disruption RNA has not been more extensively exploited.

As an example, we designed a series of oligonucleotides that bind to the important stem-loop in all RNA species in HIV, and TAR element. We synthesized a number of oligonucleotides designed to disrupt TAR, and showed that several indeed did bind to TAR, disrupt the structure, and inhibit TAR-mediated production of a reporter gene (157). Futhermore, general rules useful in disrupting stem-loop structures were developed as well.

Although designed to induce relatively nonspecific cytotoxic effects, two other examples are noteworthy. Oligonucleotides designed to bind to a 17 nucleotide loop in *Xenopus* 28S RNA, required for ribosome stability and protein synthesis, inhibited protein synthesis when injected into *Xenopus* oocytes (158). Similarly, oligonucleotides designed to bind to highly conserved sequences in 5.8S RNA inhibited protein synthesis in rabbit reticulocyte and wheat germ systems (159).

OCCUPANCY-ACTIVATED DESTABILIZATION RNA molecules regulate their own metabolism. A number of structural features of RNA are known to influence stability, various processing events, subcellular distribution, and transport. As RNA intermediary metabolism is better understood, many other regulatory features and mechanisms will probably be identified.

5' Capping A key early step in RNA processing is 5' capping (Figure 1). This stabilizes pre-mRNA and is important for the stability of mature mRNA. It also is important in binding to the nuclear matrix and nuclear transport of mRNA. As the structure of the cap is unique and understood, it presents an interesting target.

Several oligonucleotides that bind near the cap site have been shown to be active, presumbly by inhibiting the binding of proteins required to cap the RNA. Again, however, this putative mechanism has not been rigorously demonstrated in any published study. In fact, none of the oligonucleotides have been shown in any published study to bind to the sequences for which they were designed. For example, the synthesis of SV40 T-antigen was reported to be most sensitive to an oligonucleotide linked to polylysine and targeted to the 5' cap site of RNA (160).

In studies in our laboratory, we have designed oligonucleotides to bind to 5' cap structures and reagents to specifically cleave the unique 5' cap structure (161).

Inhibition of 3' polyadenylation In the 3' untranslated region of pre-mRNA molecules, there are sequences that result in the post-transcriptional addition of long (hundreds of nucleotides) tracts of polyadenylate. Polyadenylation stabilizes mRNA and may play other roles in the intermediary metabolism of RNA species. Theoretically, interactions in the 3' terminal region of pre-mRNA could inhibit polyadenylation and destabilize the RNA species. Although there are a number of oligonucleotides that interact in the 3'' untranslated region and display antisense activities, to date no study has reported evidence for alterations in polyadenylation.

Other mechanisms In addition to 5' capping and 3' adenylation, clearly other sequences in the 5' and 3' untranslated regions of mRNA affect the stability of the molecules. Again, a number of antisense drugs may work by these mechanisms.

Zamecnik & Stephenson (1) reported that a 13-mer targeted to untranslated 3' and 5' terminal sequences in Rous sarcoma viruses was active. Oligonucleotides that were conjugated to an acridine derivative and targeted to a 3'-terminal sequence in type A influenza viruses were reported to be active (109, 162, 163). Against several RNA targets, studies in our laboratories have shown that sequences in the 3' untranslated region of RNA molecules are often the most sensitive. For example, ISIS 1939, a 20-mer phosphorothioate that binds to and appears to disrupt a predicted stem-loop structure in the 3' untranslated region of the mRNA for ICAM, is a potent antisense inhibitor. However, inasmuch as a 2'-O-methyl analog of ISIS 1939 was much less active, it is likely that in addition to destabilization to cellular nucleolytic activity, activation of RNase H (see below) is also involved in the activity of ISIS 1939 (164).

ACTIVATION OF RNase H RNase H is an ubiquitous enzyme that degrades the RNA strand of an RNA-DNA duplex. It has been identified in organisms as diverse as viruses and human cells (for review see 165). At least two classes of RNase H have been identified in eukaryotic cells. Those in yeast and multiple enzymes with RNase H activity have been observed in prokaryotes (165). Furthermore, data suggest that there are multiple isozymes in eukaryotic cells.

Although RNase H is involved in DNA replication, it may play other roles in the cell and is found in the cytoplasm as well as the nucleus (166). The concentration of the enzyme in the nucleus is thought to be greater, however, and some of the enzyme found in cytoplasmic preparations may be due to nuclear leakage.

RNase H activity is quite variable. It is absent or minimal in rabbit reticulocytes (167) but present in wheat germ extracts (165) in a wide range of

cells (16). The level of RNase H varies as a function of development, differentiation, and rate of cell division (165). In HL60 cells, for example, the level of activity in undifferentiated cells is greatest; it is relatively high in DMSO and vitamin D–differentiated cells, and much lower in PMA-differentiated cells (G. D. Hoke et al, unpublished observations).

The precise recognition elements for RNase H are unknown; however, it has been shown that oligonucleotides with DNA-like properties as short as tetramers can activate RNase H (168). Changes in the sugar influence RNase H activation, as sugar modifications that result in RNA-like oligonucleotides, e.g. 2'-fluoro or 2'-O-methyl, do not appear to serve as a substrate for RNase H (44, 169). Alterations in the orientation of the sugar to the base can also affect RNase H activation, as α-oligonucleotides are unable to induce RNase H or may require parallel annealing (41, 170). Additionally, backbone modifications influence the ability of oligonucleotides to activate RNase H. Methylphosphonates are not substrates for RNase H (74, 143). In contrast, phosphorothioates are excellent substrates (106, 155, 171; G. D. Hoke unpublished observations). More recently, chimeric molecules have been studied as substrates for RNase H (172, 173). A single ribonucleotide in a sequence of deoxyribonucleotides was recently shown to be sufficient to serve as a substrate for RNase H when bound to its complementary deoxyoligonucleotide (174).

Despite the information about RNase H and the demonstrations that many oligonucleotides may activate RNase H in lysate and purified assays (168, 175–177), relatively little is known about the role of structural features in RNA targets in activating RNase H. There is little direct proof that RNase H activation is, in fact, the mechanism of action of oligonucleotides in cells. Recent studies in our laboratories provide additional, albeit indirect, insights into these questions. ISIS 1939 is a 20-mer phosphorothioate complementary to a sequence in the 3' untranslated region of ICAM-1 RNA. It inhibits ICAM production in human umbilical vein endothelial cells, and northern blots demonstrate that ICAM-1 mRNA is rapidly degraded. A 2'-O-methyl analog of ISIS 1939 displays higher affinity for the RNA than the phosphorothioate, is stable in cells, but inhibits ICAM-1 protein production much less potently than ISIS 1939. It is likely that ISIS 1939 destabilizes the RNA and activates RNase H. In contrast, ISIS 1570, an 18-mer phosphorothioate that is complementary to the translation initiation codon of the ICAM-1 message, inhibited production of the protein but caused no degradation of the RNA. Thus, two oligonucleotides that are capable of activating RNase H had different effects, depending on the site in the mRNA to which they bound (164).

COVALENT MODIFICATION OF THE TARGET NUCLEIC ACID BY THE OLIGONUCLEOTIDE A large number of oligonucleotides conjugated to alkylating and photoactive alkylating species have been synthesized and tested for effects on purified nucleic acids and intracellular nucleic acid targets (162, 178). The potential advantage of such modifications is, of course, enhanced potency. The potential disadvantages are equally obvious: nonspecific alkylation in vivo and resulting toxicities.

A variety of alkylating agents have been used to modify single-stranded DNA covalently and have been shown to induce alkylation at sequences predicted by the complementary oligonucleotide to which they were attached (178–182). Similar alkylators have been employed to modify double-stranded DNA covalently after triplex formation (125, 137, 183, 184).

Photoactivatable crosslinkers and platinates have been coupled to oligonucleotides and shown to crosslink sequence-specifically as well. Photoactivatable crosslinkers coupled to phosphodiesters, methyl-phosphonates, and phosphorothioates have been shown to produce sequence-specific crosslinking (59, 130, 185–190). Photoreactive crosslinking has also been demonstrated for double-stranded DNA after triplex formation (136, 191).

Preliminary data suggesting that covalent modifications of nucleic acids in cells is feasible and may enhance the potency of oligonucleotides have also been reported. Psoralen-linked methylphosphonate oligonucleotides were reported to be significantly more potent than methylphosphonate oligonucleotides in inhibiting rabbit globin mRNA in rabbit reticulocyte lysate assay (33). Psoralen-linked methylphosphonates were also reported to be more potent in inhibiting herpes simplex virus infection in HeLa cells in tissue culture (147). Additionally, although it did not produce covalent modification, a 9-mer phosphodiester conjugated with an intercalator inhibited mutant Ha-ras synthesis in T-24 bladder carcinoma cells (81).

OLIGONUCLEOTIDE-INDUCED CLEAVAGE OF NUCLEIC ACID TARGETS Another attractive mechanism by which the potency of oligonucleotides might be increased is to synthesize derivatives that cleave their nucleic acid targets directly. Several potential chemical mechanisms are being studied, and positive results have been reported.

The mechanism that has been most broadly studied is to conjugate oligonucleotides to chelators of redox-active metals and generate activated oxygen species that can cleave nucleic acids. Dervan and colleagues have developed EDTA-conjugated oligonucleotides that cleave double-stranded DNA sequence specifically after triplex formation (124, 137). Dervan and others have employed EDTA-oligonucleotide conjugates to cleave single-stranded DNA (192, 193). It is thought that EDTA chelates iron, which generates hydroxyl radicals that cleave the DNA; however, the cleavage occurs at several nucleotides near the nucleotide at which EDTA is attached.

In the presence of copper, oligonucleotides that are conjugated to 2,10-phenantholine also cleave DNA with some sequence specificity (129,

133–135, 194, 195), as do porphyrin-linked oligonucleotides when exposed to light (196–198). Phorphyrin-linked oligonucleotides, however, oxidize bases and induce crosslinks as well as cleave the phosphodiester backbone.

To date, no reports have demonstrated selective cleavage of an RNA or enhanced potency of oligonucleotides in cells using oligonucleotides and cleaving moieties that employ these mechanisms. Studies in progress in a number of laboratories will probably soon explore this question.

Another mechanism that may be intrinsically more attractive for therapeutic applications, particularly for cleavage of RNA targets, is a mechanism analogous to that used by many ribonucleases, nucleotodiyltransferases, phosphotransferases, and ribozymes.

Ribozymes are oligoribonucleotides or RNA species capable of cleaving themselves or other RNA molecules (199). Furthermore, the Tetrahymena ribozyme has been shown to cleave DNA, but at a slower rate than RNA (200). Although several classes of ribozymes have been identified that differ with regard to substrate specificity, the use of internal or external guanosine, and other characteristics, they all employ similar enzymatic mechanisms. Cleavage and ligation involve a Mg^{2+}-dependent transesterification with nucleophilic attack by the 3'-hydroxyl of guanosine (200).

The notion that we might design a relatively small ribozyme that could interact with desired sequences as a therapeutic was given impetus by studies that showed activity for ribozymes as short as a 19-mer (201) and by the demonstration that ribozyme activity can be retained after substitutions such as phosphorothioates are introduced (200).

Other approaches to creating oligonucleotides that cleave RNA targets are to synthesize oligonucleotides with appropriate adducts positioned to catalyze degradation via acid-base mechanisms. Substantial progress is being made in this area as well (P. D. Cook et al, unpublished observations).

A few studies have attempted to compare activities of oligonucleotides targeted to different receptor sequences in the same RNA. In our laboratories, we have shown that the most sensitive site in ICAM mRNA appears to be the 3' untranslated region (164). Against PLA_2, the most active molecules are also directed to sequences in the 3' untranslated region. In contrast, the most active molecules against ELAM are in the 5' untranslated region (C. F. Bennett et al, unpublished observations). However, oligonucleotides directed to the 5' cap site, translation initiation codon, and coding regions have also shown activity (for review see 202, 203).

In conclusion, an array of potential post-binding mechanisms have already been identified for oligonucleotides. For specific oligonucleotides, however, insufficient data are available to draw firm conclusions about mechanisms. More than one mechanism may very well play a role in the activity of a given oligonucleotide. Many additional mechanisms are likely to be identified as

progress continues. It is important to consider the structure and function of receptor sequences in designing oligonucleotides and to continue to study potential mechanisms in detail. Clearly, RNase H may play a role in the mechanisms of many oligonucleotides, but, equally clearly, it is not critical for the activity of others. In the future, the mechanisms for which oligonucleotides are designed will probably be optimized for each target and class of oligonucleotide.

MEDICINAL CHEMISTRY

The core of any rational drug discovery program is medicinal chemistry. Although the synthesis of modified nucleic acids has been a subject of interest for some time, the intense focus on the medicinal chemistry of oligonucleotides dates perhaps to no more than three years prior to this writing. Consequently, the scope of medicinal chemistry has recently expanded enormously, but the biological data to support conclusions about synthetic strategies are only beginning to emerge. As several excellent reviews have been published recently, I focus here strictly on design features and progress in evaluating various approaches to enhance the properties of oligonucleotides as drugs (12, 16, 202, 203).

Modifications in the base, sugar, and phosphate moieties of oligonucleotides have been reported. The subject of medicinal chemical programs include approaches to create enhanced affinity and more selective affinity for RNA or duplex structures; the ability to cleave nucleic acid targets; enhanced nuclease stability, cellular uptake, and distribution; and in vivo tissue distribution, metabolism and clearance.

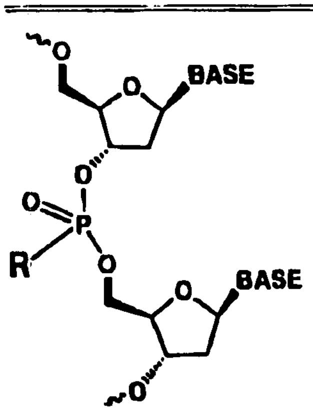
Modifications of the Phosphate

Table 6 shows the structures of various phosphate analogs. The properties of phosphodiester, phosphorothioate, and methylphosphonate analogs have been discussed extensively. More recently, phosphorodithioates have been synthesized and reported to be nuclease-resistant and to form stable duplexes with complementary DNA (204). Of course, a potential advantage of the phosphorodithioates is the lack of a chiral center. Another interesting recent modification is the replacement of one of the nonbonding oxygen atoms with a borane group (205). A dimer was reported to be nuclease-resistant, but little additional information is available.

Modifications of the Pentofuranose Linker

Modifications of oligonucleotides that replace phosphorous may be attractive because they support the design of oligonucleotides that may have a range of

Table 6 Phosphate modifications and analogs



Structure	R	Name
1	O^-	Phosphodiester
2	S^-	Phosphorothioate
3	Me	Methyl phosphonate
4	N (alkyl)	Phosphoramidate
5	S and $O \rightarrow S$	Phosphorodithioate
6	H_3B	Boranophosphate

charges from nonionic to negatively or positively charged. In theory, reducing the anionic character of oligonucleotides may enhance hybridization and pharmacokinetic properties.

As discussed above, earlier modifications were not specifically directed to the needs of oligonucleotide drugs. Recent modifications have attempted to maintain the geometry and spacing required to support hybridization. Replacement of the phosphorous dioxygen moiety with a methylene group has been reported by two groups (51–53). This "formacetal" linkage forms a stable duplex with DNA and to be nuclease-resistant, but it is not amenable to additional modifications without creating chiral centers and would result in a water-insoluable molecule if fully substituted throughout an oligonucleotide. Other one-for-one substitutions have been reported, but the substitutions are either less interesting or have not been evaluated sufficiently to determine their potential (for review see 12).

More complex substitutions have also been reported recently, including two atom substitutions in which the phosphorous and 5' oxygen atoms were replaced by a sulfonamide linkage (54) or methyl sulfoxyl linkage (206). A thymine tetramer in which the phosphate backbone structure was replaced with dimethylene sulfonate was recently found to hybridize with natural oligonucleotides (207). Additionally, an acetamide group has been substituted for the backbone phosphate structure in a dinucleoside (207). Finally, a great many other substitutions in the backbone have been made and will probably be published in the next year, so the repertoire of compounds should continue to increase.

Pentofuranose (Sugar) Modifications

A significant number of modifications have recently been reported. In essence, these derive from two strategies with different objectives. Oligonucleotides in which the sugars are modified uniformly throughout are designed to enhance affinity to RNA targets by facilitating the formation of a more stable helix. They also may enhance nuclease stability and membrane permeability, but these outcomes are usually secondary to the hybridization goals. In contrast, pendant modifications have also been synthesized primarily to enhance pharmacokinetics or to introduce alkylating or cleaving moieties. In any event, the sugar at the 2' position is clearly an attractive site for medicinal chemistry. Some of the properties of a few of the 2' modified oligonucleotides are shown in Table 3. This remains a fertile area for medicinal chemistry, and additional advances are likely.

More substantive alterations in sugar and even replacement of sugar are also possible. Of course, α-oligonucleotides represent one type of modification (41), but numerous other approaches are feasible. Carbocyclic (49, 50) and acyclic (47, 48) structures have been reported.

Heterocyclic Modifications

Numerous heterocyclic modifications have also been described. Many of these have been designed to enhance affinity and/or alter specificity (224). Other modifications have been developed to attach pendant modifications that may alkylate, intercalate, or cleave, as well as others that may enhance pharmacokinetic properties.

Conclusions

In conclusion, it is clear that an enormous scope for medicinal chemistry exists and that the major programs are already beginning to pay dividends.

ACTIVITIES OF OLIGONUCLEOTIDES

In the past several years, scores of articles have been published demonstrating the activity of a large number of oligonucleotides in a variety of systems. A number of excellent reviews have summarized the activities of these compounds (16, 202, 203, 208). The activities of oligonucleotides in assays of cell-free protein synthesis and after injection into cells of several types have also been summarized. Consequently, I provide a brief summary of the activities of oligonucleotides in cell-based assays and a comment on the limited in vivo data reported to date.

Activities in Cells in Tissue Culture

To date, oligonucleotides have been found to inhibit the growth of a large number of viruses in tissue culture, the expression of numerous oncogenes, a variety of normal cellular genes, and a number of transfected reporter genes controlled by several regulatory elements. The oligonucleotides used, the cells employed, and the receptor sequences, concentrations, and conditions have differed widely. Only a few of the studies have reported detailed dose response curves and conditions. Studies for which sufficient information was presented are summarized in Table 7.

The data presented in Table 7 support only a few generalizations, as follows:

1. Even though phosphodiesters are relatively rapidly degraded, a number of laboratories have reported activities for unmodified phosphodiester oligonucleotides in cells incubated in the absence of serum. The concentrations required to display activity were typically greater than 10 μM.
2. A variety of modified oligonucleotides have been reported to be active. Methylphosphonates appear to be less potent than phosphorothioates, but considerable variation has been noted, depending on the system. Conjugation of alkylators and intercalators to phosphodiesters and methylphosphates increases potency. Lipophilic and poly-lysine conjugates have also displayed enhanced activities.
3. Oligonucleotides have demonstrated a broad array of activities against viral targets, oncogenes, normal host gene products, and various transfected genes. Thus, clear evidence supports the broad, potential applicability of these drugs.
4. Although the data from studies incorporated in Table 7 are limited, when combined with the in vitro toxicologic data, the therapeutic indexes of phosphorothioates appear to be quite high in vitro. Methylphosphonates appear to have lower therapeutic indexes. Too few data are available to draw conclusions about other classes of oligonucleotides.
5. Very few data support putative mechanisms of action, and generalizations concerning desired mechanisms of action are not possible. Nevertheless, a variety of mechanisms of action may be employed by oligonucleotides to result in significant biological activities.

In Vivo Activities

Two earlier investigations have suggested in vivo activities of antisense drugs against viral infections. Although no data were reported, a methylphosphonate oligonucleotide was indicated to have been active in a mouse model of herpes simplex virus I infection (147). Another study claimed in vivo activity against tick-borne encephalitis virus (213).

Table 7 Summary of antisense oligonucleotide Activities (in vitro)'

Target	Cell type	Serum	Oligo types	Length	Concentration	Reference
Viruses						
HTLV-III	H9 cells	−	P	12–26	5-50 mg/ml	148
HIV	H-T cells	+	PS	14–28	0.5 1 μM	20
HIV (gag/pol)	H-T cells	+	PS	18-24	1-10 μM	211
HIV	H9 cells	−	PS, others	20	4-20 μg/ml	19
HIV	CZM cells	+	PS	18–28	10 μM	157
Herpes simplex	Vero cells	+	CH_3P	7	50-100 μM	98
Herpes simplex	HeLa cells	+	PS	28	1–10 μM (non-antisense)	76
Herpes simplex	Vero cells	+	CH_3P	12	20-50 μM	15
Herpes simplex	Vero cells	+	CH_3P psoralen	12	5 μM	15
Herpes simplex	HeLa cells	+	PS	21	0.2-4 μM	155
Vesicular stomatitis	L929 cells	+	CH, P	9	25-50 μM	99
Vesicular stomatitis	L929 cells	+	P-lipid	11	50-150 μM	85
Vesicular stomatitis	L929 cells	+	P-poly l-lysine	10–15	0.1 μM	212
Influenza	MDCK cells	+	P-acridine	11	50 μM	109
Tick-borne encephalitis	—	+	Various	Var.	0.1-1 μM	213
SV40	MDCK cells	+	CH_3P	6–9	25 μM	214
Rous	Chicken fibroblasts	+	Various	Var.	10 μM	1
Hepatitis B	Alexander	+	P	15	8.5 μM	215
Bovine papilloma virus	C-127 cells	+	PS	4-30	0.01-1 μM	209
Oncogenes						
c-myc	T-lymphocytes	+	P	15	30 μM	96
c-myc	HL-60 cells	+	P, PS	15	10 μM	216
c-myc	Burkitt cells	−	P	21	100 μM	146

c-myb	PMBC	+	P	18	40 mg/ml	217
BCL-2	L697 cells	−	P, PS	20	25–150 μM	97
N-myc	Neuroblastoma cells	+	P	15	1–5 μM	155
N-ras	T15 cells	+	CH_3P	9	Inactive	100
Host genes						
Multiple drug Resistance	MCF-1 cells	+	PS	15		218
PCNA (cyclin)	3T3	+	P	18	30 μM	219
Prothymosin	Human myeloma cells		P	22		153
T cell receptor	T cells	+	P	22		153
Gm CSF	Endothelial cells	−	P	15, 18	10^{-8}M	153
CSF-1	FL-*ras/myc* cells	+	P	?	?	220
EGF receptor		+	P	13	30 μM	221
BFGF	Human astrocytes	−	P	15	10-75 μM	229
β Globin	Rabbit reticulocytes	+	CH_3P	9	100 μM	222
TAU	Neurons	−	P	20-25	3-50 μM	228
CAMP-Protein kinase II β	HL-60 cells	+	P	21	15 μM	210
Myeloblastin	HL-60 cells	+	P	18	?	227
Phospholipase A_2 activating protein	BC3H,	+	P	25	25 μM	233
ICAM-1	A549 HVEC lymphocytes	−	PS	18-20	0.01-1 μM	154
IL-2	T-lymphocytes	−	P	15	5 μM	72
IL-1a	HUVEC	+	P	18	10 μM	154
IL-1β	Monocytes	+	PS	15	0.1-2.5 μM	105

Table 7 (*Continued*)

Target	Cell type	Serum	Oligo types	Length	Concentration	Reference
IGF-1	Myoblasts	−	P	15	10 μM	225
Perforin	T-lymphocytes	−	P	18	5-35 μM	226
Other						
Chloramphenicol acetyl transferase	CV-1 cells	+	P, PS, CH_3P	21	5-30 μM	75
Placental alkaline phosphatase driven by HIV TAR	SK-mel-2 cells	+	PS	18-28	0.25-5 μM	157
Chloramphenicol Acetyl transferase Driven by human papilloma virus E2 responsive element	C-127 and CV-1 cells	+	PS	14–20	1–10 μM	209

[a] Abbreviations: cAMP, cyclic AMP; CH_3-P, methylphosphonate oligonucleotides; EGF, epidermal growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; HB, hepatitis B; HIV, human immunodeficiency virus; HSV, herpes simplex virus; HTLV, human T-cell lymphotrophic virus; IV, influenza virus; P, phosphodiester oligonucleotides; P-acridine, phosphodiester oligonucleotide conjugated with acridine moiety; P-lipid, phosphodiester oligonucleotide conjugated with lipid moiety; P-S, phosphorothioate oligonucleotides; PCNA, proliferating cell nuclear antigen; PMA, phorbol myristic acid; RSV, Rous sarcoma virus; TAR, Tat response element; TBE, tick-borne encephalitis; VSV, vesicular stomatitis.

Topical application of ISIS 1082 in an aqueous buffer to the cornea of mice infected with herpes virus 1 inhibited viral growth in a concentration-dependent fashion and cured the infection at concentrations greater than 1% (230). The activity of ISIS 1082 was equivalent to trifluorothymidine and was associated with no local or systemic toxicities.

CONCLUSIONS

Oligonucleotides designed to interact with nucleic acid receptors represent a potentially revolutionary advance in pharmacotherapy. Advances in the recent past and the intense, current focus assure that the paradigm will be fully explored.

Oligonucleotides have already been shown to work in vitro and have proven to be invaluable pharmacologic tools. The progress in resolving the basic pharmacological questions relating to oligonucleotide therapeutics and in resolving issues that will influence the commercialization of new drugs of this class has been impressive. Moreover, advances in medicinal chemistry are exciting and argue that exciting new classes of these drugs are forthcoming.

Much remains to be learned; a great deal remains to be accomplished before the paradigm is fully proven and the opportunity it represents realized. In the coming years, the key tasks will be (*a*) to place oligonucleotide therapeutics on a solid pharmacological footing by performing careful dose response curves in well-designed experiments, and (*b*) to advance the development of oligonucleotide pharmaceutic products to the point that the paradigm can be tested in man.

There is now cause for considerable optimism that the promise of oligonucleotide therapeutics may be realized.

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PHARMACOLOGY OF PROTEIN KINASE INHIBITORS

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KEY WORDS: cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinase C, Ca^{2+}/calmodulin kinase II inhibitor, protein phosphorylation

INTRODUCTION

A complete understanding of the organization and functioning of the second messenger system requires the expertise and cooperation of several different scientific disciplines, such as molecular pharmacology, genetic manipulation, biochemistry, and cell biology. The advent of a new class of effective pharmacological agents is always an event of considerable interest, in particular when this class consists of new types of antagonists that act by specifically blocking one or more of the steps in intracellular signaling systems (1). Although various aspects of protein-phosphorylation systems have been investigated, uncertainties concerning the complex cellular responses in the second messenger system remain (2–4). Improved and sophisticated methods must be designed to estimate changes in the activities of cellular response elements after extracellular stimuli. While our comprehension of the biochemistry and molecular biology of protein kinases has progressed, the function of these enzymes in intact cells has been much more difficult to understand. For this reason researchers studying second messenger systems have long sought the development of specific and effective protein kinase inhibitors that would permit the definitive determination of the physiological role of the protein kinases (5). Protein kinase inhibitors can be used to determine the physiological significance of the protein phosphorylation systems in various types of cells. To elucidate the physiological function of each

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Disposition of the ^{14}C-Labeled Phosphorothioate Oligonucleotide ISIS 2105 after Intravenous Administration to Rats

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ABSTRACT

5′-TTGCTTCCATCTTCCTCGTC-3′ (ISIS 2105) is a phosphorothioate oligodeoxynucleotide currently being evaluated as an intralesional antiviral drug for the treatment of genital warts that are caused by the human papillomavirus. ISIS 2105, labeled with ^{14}C (at the carbon-2 position of thymine) was administered as a single i.v. injection (3.6 mg/kg) to female Sprague-Dawley rats to assess the disposition of the drug. After i.v. administration of [^{14}C]2105, blood radioactivity disappeared in a multiexponential manner with the half-lives of the phases equal to 0.4, 1.9, 7.1 and 5.1 hr. The initial volume of distribution was 22 ml and the postdistribution volume of distribution was 1076 ml, which indicated an extensive distribution of radioactivity. The apparent blood clearance was 14.7 ml/hr. The radioactivity in the expired air accounted for 51% of the administered dose over the 1 O-day period. Urinary and fecal radioactivity accounted for 15% and 5% of the administered dose, respectively. The major sites of radioactivity uptake were the liver (up to 22.8% of the dose), kidneys (renal cortex, up to 14% of the dose), bone marrow (up to 14% of the dose), skin (up to 13% of the dose) and skeletal muscle (up to 9% of the dose). Other tissues contained approximately 1% or less of the dose. The overall recovery of radioactivity 10 days postdosing was 95.1 ± 7.5% (mean ± SD.) of the administered single dose. The radioactivity in the blood was almost completely in the plasma during the course of the study. In the plasma, the radioactivity was extensively bound to proteins, as assessed by size-exclusion high-performance liquid chromatography (HPLC), in samples up to 8 hr postdosing. Retention data on size-exclusion HPLC and in vitro incubations using purified proteins suggested that the plasma proteins that bound [^{14}C]2105 were albumin and α_2-macroglobulin. The complex formed between the plasma proteins and [^{14}C]21 05derived radioactivity was dissociated on anion-exchange HPLC to indicate that the great majority of plasma radioactivity coeluted with intact [^{14}C]21 05 in samples that contained sufficient radioactivity for analysis. There was a timedependent decrease in the proportion of hepatic and renal radioactivity that coeluted with the intact [^{14}C]2105 during the course of the study. The urine did not contain radioactivity that eluted with intact [^{14}C]2105 on anion-exchange HPLC.

Substantial interest in the development of oligonucleotide-based therapeutic agents has been generated (Zamencik et al., 1978; Stein et al., 1988; Mirabelli et al., 1991; Crooke, 1992). Several first-generation oligonucleotide analogs, in which one or more of the substituents on the internucleotide phosphate are modified, e.g., phosphorothioates, methylphosphonates and phosphorodithioates, have been synthesized and tested (Matsukura et al., 1987; Crooke, 1992). Each of these modifications was shown to enhance the nuclease stability of oligonucleotides significantly (Agrawal and Goodchild, 1987; Crooke, 1991).

Phosphorothioate oligodeoxynucleotides have been studied extensively as potential antisense therapeutic agents. They displayed potent antiviral activities and inhibitory activities against a wide range of mammalian gene products (Mirabelli et al., 1991; Crooke, 1992). Although phosphorothioate oligodeoxynucleotides may display pharmacological activities that are the result of mechanisms other than antisense, [redacted] (Mirabelli et al., 1991; Crooke, 1992). Optimal antisense activities for phosphorothioate oligodeoxynucleotides are usually observed with oligonucleotides that are 18 to 21 nucleotides in length (Cohen, 1989).

Phosphorothioate oligodeoxynucleotides have been shown to be stable (half-lives > 24 hr) in serum, cell homogenates, cells, cerebrospinal fluid and organs (Crooke, 1991; Campbell et al., 1990; Agrawal et al., 1988; Crooke, 1993; Stein et *al.*, 1988; Loke et *al.*, 1989; Hoke et al., 1991). They were taken up by many types of cells in tissue culture (for review, see Crooke, 1991; Crooke, 1993) and cellular uptake and in *vivo* activities can sometimes be enhanced by cationic lipids (Bennett et *al.*, 1992; Perlaky et al., 1993).

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ABBREVIATIONS: HPLC, high-performance liquid chromatography; SE-HPLC, size-exclusion HPLC; SAX-HPLC, strong anion-exchange HPLC; ISIS2105, 5′-TTGCTTCCATCTTCCTCGTC-3′; AUC, area under the plasma curve.

Two previous studies investigated the pharmacokinetics of phosphorothioate oligodeoxynucleotides in animals. The pharmacokinetics of a 20-mer phosphorothioate oligodeoxynucleotide were determined after single i.v. or i.p. doses of 30 mg/kg. The oligonucleotide was labeled at each internucleotide linkage with ^{35}S. The compound was shown to be bioavailable after i.p. administration to have broad peripheral tissue distribution and to be cleared primarily by renal excretion. Gel electrophoretic analysis demonstrated significant, albeit slow, metabolism in the liver, kidney and intestines (Agrawal et al., 1991). A 27-mer phosphorothioate deoxyoligonucleotide labeled with ^{35}S was reported to display biexponential elimination in plasma with an elimination half-life in excess of 40 hr after a single i.v. dose of 4.8 mg/kg in the rat (Iversen, 1991).

ISIS 2105 is a phosphorothioate deoxyoligonucleotide that is active against human papillomaviruses (Cowsert et *al.*, 1993). It is currently undergoing pivotal Phase II clinical trials for the treatment of genital warts. There were two objectives of the current study. First, we wanted to develop and evaluate a radiolabeling method that results in higher-specific-activity oligonucleotides to support more detailed pharmacokinetic studies and a more definitive evaluation of metabolism that could be used in clinical trials. Second, we sought to perform more definitive pharmacokinetic distribution and metabolic studies in which metabolites in the plasma, urine and tissues were evaluated using HPLC techniques that support more quantitative analyses.

To achieve these objectives, we radiolabeled ISIS 2105 at the carbon-2 position of all thymidines. Because thymidine is metabolized into CO_2, metabolism can be measured by collecting expired air. This provides an estimate of the total metabolism of the oligonucleotide. Coupled with the extraction of radioactivity and HPLC analysis, a reasonably precise evaluation of metabolism can be achieved. Obviously, a full evaluation of metabolism will require the analysis of intermediates between the intact oligonucleotide and CO_2. We also developed HPLC methods to evaluate the integrity of the radiolabel in tissues and biological fluids.

Materials and Methods

ISIS 2105 Synthesis and Purification

The ^{14}C-labeled phosphorothioate ISIS 2105 was chemically synthesized by using the deoxynucleoside phosphoramidite approach (Beaucage and Caruthers, 1981; Matteucci and Caruthers, 1981). The phosphorothioate linkage was generated by oxidizing with ^{3}H-1,2-benzodithiole-3-one-1,1-dioxide (Iyer et *al.*, 1990) instead of aqueous iodine. All reagents and materials for the solid-phase synthesis of DNA were purchased from commercial sources with the exception of the ^{14}C-thymidine phosphoramidite. The ^{14}C-labeled phosphoramidite synthon was generated from ^{14}C-thymidine (^{14}C at the carbon-2 position of the thymine ring (specific activity, ~56.3 mCi/mmol, Sigma, St. Louis, MO) as described elsewhere (D. Dellinger and H. Sasmor, manuscript in preparation).

The crude synthetic oligonucleotide was purified by trityl-on reverse-phase HPLC by using a methanol gradient in a 0.25 M sodium acetate mobile phase buffer. The HPLC product was acid deprotected and recovered by ethanol precipitation as the sodium salt. The final product was analyzed by using electrophoresis with 20% denaturing polyacrylamide gels and the full length integrity (88% full length material) and the radiochemical purity (88% of counts per minute in the full length product) was determined by laser scanning densitometry and quantitative phosphorimaging (Molecular Dynamics, Foster City, CA), respectively. The specific activity of the final product was ~2.0 × 10^{8} cpm/μmol and had approximately 97.6% phosphorothiodiester content (vs. 2.4% phosphodiester) as determined by high-field nuclear magnetic resonance (500 MHz, University of Missouri, Columbia, MO).

Formulation of ^{14}C-ISIS 2105 in phosphate-buffered saline. We formulated 2.22 × 10^{8} cpm (11.7 pmol) of the purified product by dilution in phosphate-buffered saline, pH 7.0, (Irvine Scientific, Pasadena, CA) to deliver 2 × 10^{7} cpm per 100-μl injection. The solution was sterile filtered through a 0.22-μm cellulose acetate filter (S&S Uniflow, Keene, NH) and the radioactivity was determined in 100 μl by aqueous scintillation.

Animals

Young adult female Sprague-Dawley rats (9-10 weeks old, 175-209 g at the time of randomization) were purchased from Taconic Farms (Germantown, NY). The animals were acclimated to the surroundings of the animal facility used for radioactive studies for approximately 1 week before dosing and were examined by a veterinarian before they were assigned to the study. During acclimation, the rats were housed in individual stainless steel suspended cages with noncontact bedding (Cellu-dri, Shepherd Specialty Papers, Kalamazoo, MI). Twenty-four hours before dosing, the rats to be used for urine, feces and expired air collection were transferred to Nalgene metabolism cages (Nalge, Rochester, NY). At the time of dosing, the animals used for the collection of urine, feces and expired air were transferred to glass metabolism cages (Vanguard International, Neptune, NJ). The animals used for the collection of retro-orbital blood samples, but not for urine and feces, were housed individually in stainless steel suspended cages. Each cage was labeled with the animal identification number. Food and water were allowed *ad libitum*. The food consisted of Purina Standard Rodent Chow (#5001, Raltech Scientific Services, St. Louis, MO) in pellet form. The water was city tap water.

Compound Administration

The [^{14}C]2105 was administered in solution in phosphate buffer, pH 7, to rats by i.v. injection (100 μl) into the caudal tail vein. The concentration of [^{14}C]2105 was 1.2 mM; therefore, the dose level was approximately 3.6 mg/kg. The actual doses administered were calculated by using the assayed concentration of radioactivity in a 100-μl volume of the formulation. The [^{14}C]2105 was administered to 25 female rats. Five rats were used for the collection of urine, feces, expired air and tissues at the time of sacrifice. The remaining 20 animals were used for the collection of blood at intervals after dosing and tissues at the time of sacrifice.

Sample Collection

Urine and feces. Urine and feces were collected from the five animals housed in glass metabolism cages at 0 to 4, 4 to 8, 8 to 24, 24 to 48, 48 to 72, 72 to 96, 96 to 120, 120 to 144, 144 to 168, 168 to 192, 192 to 216 and 216 to 240 hr after administration of [^{14}C]2105.

The urine and feces were frozen immediately on excretion and were kept frozen for the entire collection period. Before sacrifice, the animals were made to urinate by gentle pressure on the urinary bladder and this urine was combined with the last sample collected. A thorough cage wash with water was performed at the time of sacrifice. At sacrifice, the blood and plasma were obtained as described subsequently. Urine, feces and cage washings were stored in a -20°C freezer.

Expired air. The radioactivity in the expired air was collected from the five animals housed in glass metabolism cages in a series of two traps. The traps contained 6 M KOH for the collection of $^{14}CO_2$. The radioactivity in the expired air was collected during the following intervals: 0 to 4, 4 to 8, 8 to 24, 24 to 48, 48 to 72, 72 to 96, 96 to 120, 120 to 144, 144 to 168, 168 to 192, 192 to 216 and 216 to 240 hr after the administration of [^{14}C]2105. The KOH samples were stored in a 4°C refrigerator until analysis.

Blood. Single blood samples (approximately 50–100 μl each) were obtained by retro-orbital puncture from two animals at each of the following time points: 0.5, 1.5, 3, 6, 12, 36, 56, 80, 104 and 128 hr. Capillary tubes containing blood were stored on ice until the blood was

aliquoted into combustion cones. Two animals were sacrificed at the following time points: 1, 2, 4, 8, 24, 48, 72, 96, 120 and 144 hr after dosing. At sacrifice, the animals were anesthetized with CO_2 and the blood (5 ml) was recovered by cardiac puncture and transferred into heparinized Vacutainer tubes (Becton Dickinson, Rutherford, NJ). The plasma was obtained from the blood by centrifugation and kept on ice before storage at -20°C. Animals were sacrificed by exsanguination.

Tissue. At the time of sacrifice, the liver, kidneys, spleen, lungs, brain, eyes, bone (femur), skeletal muscle (representative sample), ovaries, uterus, representative dorsal skin and carcass were collected and immediately frozen on dry ice and stored in a -20°C freezer.

Analytical Procedures

All determinations of total radioactivity in the tissues, excreta and blood were made with a Beckman LS 6000 scintillation system (Fullerton, CA).

The weight of the urine was determined. The radioactivity in duplicate samples of urine, plasma and cage rinse was determined in Scint-A XF (Packard Instrument, Downers Grove, IL). The radioactivity in duplicate samples of KOH was determined in modified Bray's solution (Nomeir et al., 1992). Feces were weighed and homogenized with distilled water (20% w/w) with a Brinkmann Polytron homogenizer (Lucerne, Switzerland).

The liver, lung, brain and spleen samples were weighed and finely minced before combustion. The skeletal muscle (representative sample), blood, uterus, eyes and ovaries were weighed but not pretreated before combustion. The bone marrow was removed from bone and weighed before combustion. The renal medulla and cortex were excised from the kidneys and aliquots were weighed before combustion. The carcasses from the animals that were used to determine mass balance and the skin (representative sample) were weighed, powdered on dry ice with a Waring (New Hartford, CT) blender and homogenized with distilled water (33% w/w) with a Brinkmann Polytron homogenizer.

Duplicate samples (total sample size allowing) of fecal homogenates, tissue minces and whole tissues were aliquoted and then underwent combustion with a Packard Tricarb oxidizer, model 307. The ^{14}C radioactivity was trapped in Carbosorb II (Packard Instrument). The recovery of the combustion system was determined on a daily basis and ranged from 97% to 100%.

Extraction of tissue radioactivity. To approximately 1 g of liver, 0.5 ml of extraction buffer (0.5% NP-40, 20 mM Tris HCl, pH 8.0, 20 mM EDTA, 100 mM NaCl and 2 mg/ml of proteinase K) was added and the tissue was homogenized using a Bessman tissue pulverizer (Spectrum, Houston, TX) followed by using the A pestle of a 7-ml Dounce-type tissue grinder (Wheaton, Millville, NJ). One kidney from each animal was dissected into the cortex and medullar regions. We combined 0.1 to 0.2 g with 0.25 ml of extraction buffer and homogenized it in a 1-ml Dounce-type tissue grinder (Wheaton) by using the A pestle. All samples were then incubated at 65°C for 24 hr, followed by centrifugation at 16,000 × g at 4°C for 15 min. The supernatants were removed and stored frozen at −70°C until analysis.

All samples were filtered through an Ultrafree-MC 0.22-μm filter (Millipore, Bedford, MA) at 4°C. Before analysis, the liver and kidney samples were thawed and unlabeled ISIS 2105 was added to a final concentration of 100 μM. The plasma samples were thawed at room temperature and immediately analyzed.

HPLC. SAX-HPLC was used to determine the metabolic profile of the plasma and urine and hepatic and renal tissues. Ion-exchange analyses were carried out using a Beckman System Gold liquid chromatography system with model 126 pumps, model 507 autoinjector, and model 166 detector. We analyzed 40 μl of each sample of plasma and urine, or of liver or kidney homogenate, at 260 nm on a 4.6 × 100-mm Gen Pak Fax column (Waters, Milford, MA) by using the following buffers and gradient: buffer A, 0.086 M Tris HCl, pH 8.0, 20% methanol; buffer B, 0.086 M Tris HCl, pH 8.0, 1.5 M NaBR; gradient, 0% B isocratic for 5 min and then linear to 60% B over 45 min at a flow of 0.5 ml/min. Fractions (0.5 ml) were collected and added to 5 ml of Readysafe scintillation cocktail (Beckman) and then counted in a Beckman model LS6000IC scintillation counter.

The plasma samples were also analyzed by SE-HPLC by using a Hewlett-Packard (Pasadena, CA) model 1090 M liquid chromatography system and a 7.8 × 300-mm TSK-gel G2000 SWXL column (Tosohaas, Montgomeryville, PA). The analyses were carried out in 0.05 M Na_2HPO_4, 0.1 M Na_2SO_4 and 0.05 M NaH_2PO_4, pH 7.0, at a flow of 0.45 ml/min. On-line radiochemical detection was accomplished with a Radiomatic FLO-ONE/beta model A-525A detector (Packard Instruments, Meriden, CT). The scintillation cocktail, Ultimate-Flo-V, was purchased from Packard Instruments and was used at a flow rate of 0.9 ml/min.

Calculations

The concentrations of radioactivity in the samples processed by combustion were corrected for the recovery efficiency of the combustion system, which was determined daily before the combustion of experimental samples. The observed radioactivity values were converted to compound radioequivalent concentrations. The radioequivalents were defined as the amount of parent compound, at the specific activity as administered, that would result in the observed disintegrations per minute value. Compound equivalents in a biological sample were determined by dividing the disintegrations per minute in the sample by the specific activity of the compound in disintegrations per minute per microgram. The compound equivalents were expressed in micrograms per gram of tissue and, when possible, as a percentage of the administered dose/organ or tissue. For the purpose of calculating a mean ± S.D., the tissue samples in which the radioactivity was less than twice the background for the system, the equivalents were less than 0.005 microgram equivalents per gram or the radioactivity was less than 0.005% of the dose were considered to have a value of zero.

The radioactivity in urine, feces, volatile traps and cage rinse was expressed as a percentage of the administered dose for each time interval and as a cumulative percentage. For the purposes of calculating a mean ± S.D., urine, feces, expired air and cage wash samples in which the radioactivity was less than twice the background for the system or the radioactivity was less than 0.05% of the dose were considered to have a value of zero.

Pharmacokinetic parameters for the ISIS 2105 equivalents in blood and plasma were calculated by polyexponential curve fitting of the observed concentrations, using the RSTRIP, Polyexponential Curve Fitting Program, Version 4.02 (Micromath Scientific Software, Salt Lake City, UT). The areas under the concentration-time curve and the terminal elimination half-lives for tissues were calculated by using noncompartmental analysis of the observed data (Shumaker, 1986).

Results

Percentage of the Dose of [^{14}C]2105 in Tissues

The percentage of the dose in the tissues at intervals after the single-dose administration is summarized in table 1. The maximal percentage of the dose in the liver (23%) was observed 4 hr after dosing. The maximal percentages in the renal cortex (14%) and renal medulla (3%) were observed at 4 and 8 hr after dosing, respectively. At 240 hr after a single i.v. administration, a significant portion of the radioactivity remained in the tissues. The highest percentage of the dose was observed in the renal cortex (4%), followed by the skin and bone marrow (3% each). A lesser percentage was observed in the liver (2%). All other tissues contained less than 1% of the dose. The total percentage of the dose recovered in the tissues and carcass at 240 hr was approximately 20% (table 2).

Urinary Excretion of [^{14}C]2105-Derived Radioactivity

The mean cumulative percent of [^{14}C]2105-derived radioactivity excreted in the urine is summarized in figure 1. The

TABLE 1

Percentage of dose in tissues[a] of female Sprague-Dawley rats at intervals after a single i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg

Time	Liver	Renal Medulla[b]	Renal Cortex[b]	Lung	Skeletal Muscle[c]	Bone Marrow[d]	Skin[c]
				Fraction of dose (%)			
hr							
1	16	1	7	1	9	7	9
2	20	1	10	—	7	6	13
4	23	2	14	—	5	10	12
8	19	3	12	—	2	12	6
14	14	1	13	—	1	14	9
48	10	2	10	—	2	13	2
72	8	2	10	—	—	10	11
96	6	1	8			8	8
120		1	7			8	4
144	3	1	6	—	—	5	6
240[e]	2	—	4			3	3

[a] Tissues containing ≥ 1% dose are included. Brain, spleen, ovaries, uterus and eyes contained < 1% dose at any given time.

[b] The percentages were calculated by assuming that renal cortex = 69% and renal medulla = 31% of the total kidney weight (the percentages were generated from kidney dissections in ADL MAP laboratory).

[c] The percentages were calculated from the organ weights and by assuming that muscle = 50% of the body weight and skin = 11% of the body weight (Burke et al., 1987).

[d] The percentages were calculated by assuming that bone marrow = 3% of the total body weight (Baker et al., 1979).

[e] All percentages are average data from two animals except for time 240 hr, which is the mean data from five animals.

[f] Contained < 1% of dose.

TABLE 2

Recovery of drug-related radioactivity from female Sprague-Dawley rats 246 hr after a single i.v. administration of [^{14}C]2105 at a dose of approximately 3.6 mg/kg

Sample	Animal No. 2921	2922	2923	2924	2925	Mean ± S.D.
			Dose recovered (%)			
Urine	4.5	16.2	15.3	15.0	4.1	15.1 ± 2.2
Feces	61.0	4.2	4.3	5.9	43.7	4.6 ± 0.7
Expired air		51.6	56.1	50.4	.....	51.1 ± 5.1
Tissues	16.1	21.6	16.3	22.9	20.5	20.3 ± 2.2
Cage wash	3.6	4.0	4.8	5.0	2.3	4.0 ± 1.1
Total	95.7	97.6	100.6	99.2	62.1	95.1 ± 7.5

percentage of [^{14}C]2105-derived radioactivity excreted by the urinary route was 15.1 ± 2.2%, primarily within the first 72 hr. The urinary excretion rate resulted in an elimination half-life of 55 hr (table 3).

Fecal Excretion of [^{14}C]2105-Derived Radioactivity

The mean cumulative percent of [^{14}C]2105-derived radioactivity eliminated in the feces is summarized in figure 1. The percentage of [^{14}C]2105-derived radioactivity excreted in the feces was 4.6 ± 0.7%, primarily within the first 96 hr.

Excretion of [^{14}C]2105-Derived Radioactivity in Expired Air

The excretion of [^{14}C]2105-derived radioactivity in expired air is illustrated in figure 1. The majority of the i.v. dose of [^{14}C]2105 (51.1 ± 5.1% of the dose) was eliminated by expired air, primarily within the first 96 hr. The expiration rate of [^{14}C]2105-derived radioactivity resulted in an elimination half-life of 60 hr (table 3).

Total Recovery of [^{14}C]2105-Derived Radioactivity

The total recovery of [^{14}C]2105-derived radioactivity after a single i.v. dose of [^{14}C]2105 at 3.6 mg/kg is summarized in table 2. The majority (51%) was recovered in the expired air. A smaller percentage was recovered in the urine (15%) and in the feces (4.6%). The remaining radioactivity was recovered in the tissues and carcass (20%) and the cage wash (4%). Overall recoveries ranged from 82% to 101% with a mean for the five animals of 95.1 ± 7.5%.

Pharmacokinetics of ISIS 2105 Equivalents in Blood and Tissues

The pharmacokinetic data analysis is based on microgram equivalents of ISIS 2105 present in the matrices. As such, the data describe the pharmacokinetic behavior of ISIS 2105-related radioactivity and not necessarily unchanged parent ISIS 2105.

Pharmacokinetics in blood. After i.v. administration, a peak blood radioactivity concentration of 17.2 μg equivalents/g was achieved. The concentration versus time profile in the blood is shown in figure 2, along with the fitted polyexponential curve used to calculate pharmacokinetic parameters. The blood radioactivity versus time profile was polyexponential, with four phases (table 3). The initial phase had a half-life of 0.4 hr and the terminal elimination phase had a half-life of 51 hr. The plasma data paralleled the blood elimination profile but the terminal elimination half-life was 40 hr (data not shown). At each of the corresponding time points, all or most of the radioactivity was associated with plasma and not with formed elements of blood. These data strongly suggest no binding or distribution of ISIS 2105 on or in the red blood cells. As would be expected from the long terminal half-life, the apparent blood clearance after i.v. administration was low, i.e., 14.7 ml/hr (table 3).

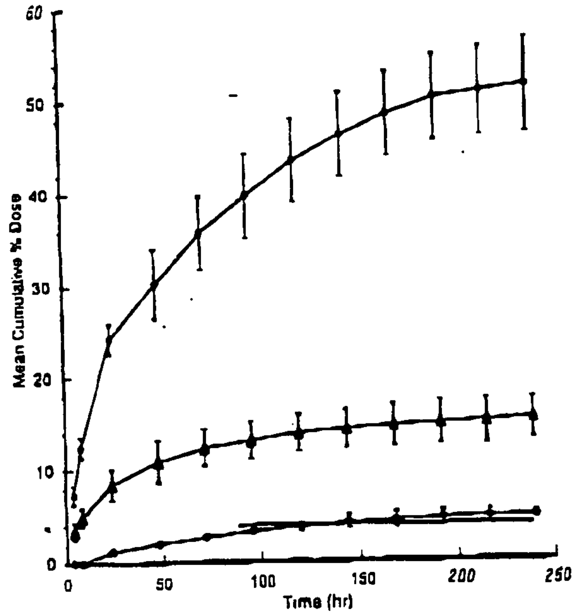

Fig. 1. Mean (± S.D., n = 4) cumulative percentage of the dose eliminated as $^{14}CO_2$ (●), in urine (▲) and in feces (◆) of female Sprague-Dawley rats after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg.

The initial volume of distribution was 22.0 ml and the post-distribution volume of distribution was 1076 ml, which indicates extensive partitioning into the tissues.

Pharmacokinetics of tissue radioactivity. The tissue radioactivity concentrations were highest in the liver, kidney, spleen and bone marrow (table 1). The elimination half-life from the liver was 62 hr, which approximated that seen in the blood. The elimination half-life was prolonged in the kidney, with observed values of 112 hr in the cortex and 156 hr in the medulla. The elimination half-life of radioactivity from the bone marrow was 78 hr (table 3).

The areas under the concentration versus time curves for the tissues (bone marrow, liver, spleen and kidney) were calculated to gauge a relative drug exposure level after i.v. administration of [^{14}C]2105. For these four tissues, comparisons were made per gram of tissue and not adjusted for the total organ weights. Of these tissues, the liver and spleen received the lowest exposure, with $AUC_{(0-\infty)}$s of 1158 and 1618 µg equivalents-hr/g, respectively. The kidney medulla received 5.6 times the liver exposure or 6497 µg equivalents-hr/g. The kidney cortex received the highest exposure, 15688 µg equivalents&/g, approximately 2.4 times the medulla and 13.5 times the liver exposure. The bone marrow exposure was approximately 2 times the liver exposure, with an AUC of 2526 µg equivalents-hr/g. These AUCs were much higher than the blood AUC; this again suggested significant partitioning of ISIS 2105 equivalents into the tissues.

Characterization of Plasma, Tissue and Urinary Radioactivity

Plasma radioactivity was present in two peaks as assessed by SE-HPLC (fig. 3). The majority of the radioactivity eluted with a retention time that was the same as that of the known complex formed between purified rat albumin and [^{14}C]2105 (approximately 18 min). A smaller proportion of the plasma radioactivity eluted with a retention time that was the same as that of the known complex formed between purified human α_2-macroglobulin and [^{14}C]2105 (approximately, 15 min). Little or no radioactivity eluted with a retention time of authentic [^{14}C]2105 (approximately 23 min). Preliminary studies (Cossum et al., manuscript in preparation) demonstrated that the affinity of ISIS 2105 for albumin and α_2-macroglobulin was in the micromolar range and that binding to both proteins was saturated when ISIS 2105 concentrations exceeded 5 to 10 µM.

To determine the integrity of ISIS 2105, plasma was applied directly to SAX-HPLC. The buffer in that system results in the extraction of radioactivity from plasma proteins. When a tissue pulverizer step was included, the total recovery of the radioactivity was approximately 60%. If only simple homogenization with a Dounce homogenizer was used, only approximately 30% of the total radioactivity was recovered. However, a comparison of the samples by HPLC revealed no differences; thus, both methods probably extract representative samples from the tissues. When plasma sampled from rats for up to 8 hr postdosing was subjected to SAX-HPLC, the majority of the radioactivity eluted with authentic [^{14}C]2105 (fig. 4). The radioactivity in peaks eluting earlier than authentic [^{14}C]2105 were, presumably, shorter metabolites of [^{14}C]2105. At the 8-hr time point, approximately 38% of the radioactivity represented intact ISIS 2105. A determination of the proportion of intact ISIS 2105 in the plasma obtained after 8 hr (*i.e.*, ≥ 24 hr) was not possible because of the low levels of radioactivity in those samples.

Figures 5 and 6 show anion-exchange radiochromatograms of extracts of hepatic and renal cortex tissues, respectively,

TABLE 3
Pharmacokinetic parameters of ISIS 2105 equivalents after a single i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg

Sample	AUC	Distribution $T_{1/2}$	Terminal $T_{1/2}$	C_{max}	T_{max}	Apparent Clearance	V_c[a]	$V_d\beta$[b]
	µg equivalents-hr/g	hr	hr	µg equivalents/g	hr	ml/hr	ml	ml
Blood	52	7.1	51	17.2	0.5	14.7	22.0	1076
Urine	—		55	—	—			
Expired air	—		80	—	—			
Liver	1158		62	19.0	4			
Renal medulla	6497		156	42.7	8			
Renal cortex	15,688		112	87.4	4			
Spleen	1618		163	10.6	1			
Bone marrow	2526		78	18.1	24			

[a] Initial volume of distribution.
[b] Postdistribution volume of distribution.

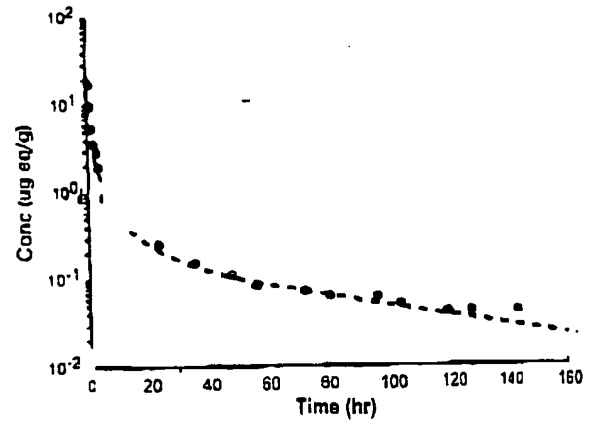

Fig. 2. Concentration of ISIS 2105 equivalents in the blood of female Sprague-Dawley rats after i.v. administration of [^{14}C]21 05 at a dose of 3.6 mg/kg. Each point represents the average of the values for two animals. The dashed line represents the line of best fit estimated from the four-compartment model.

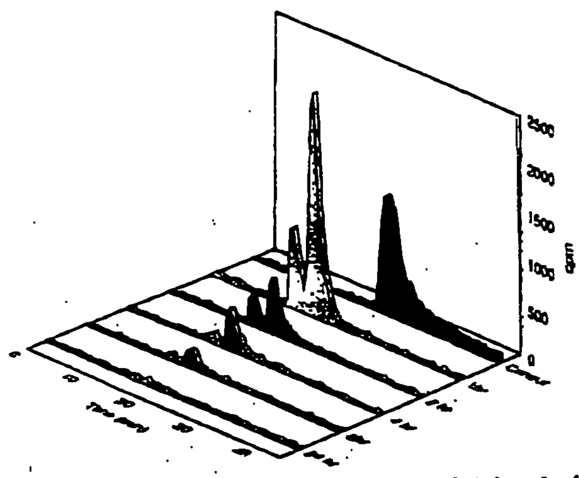

Fig. 3. SE-HPLC radiochromatograms of plasma sampled from female Sprague-Dawley rats at various times after i.v. administration of [^{14}C] 2105 at a dose of 3.6 mg/kg. The control peak represents [^{14}C]2105 that has a retention time of approximately 23 min. The plasma samples from dosed rats contained radioactive peaks eluting at approximately 15 min and approximately 16 min.

sampled at various times after the administration of [^{14}C]2105. The proportion of apparently unchanged [^{14}C]2105 decreased with time. The proportion of radioactivity present as apparently intact [^{14}C]2105 varied between the liver and kidney; [^{14}C]2105 was more stable in the kidney than in the liver. After 24 hr, approximately 15% of the radioactivity extracted from the liver represented intact ISIS 2105 and only trace levels of intact ISIS 2105 were present at 48 hr. By contrast, even after 96 hr, 48% of the radioactivity extracted from the kidney represented intact ISIS 2105.

Figure 7 shows anion-exchange radiochromatograms of urine sampled for up to 96 hr postdosing. Little or no radioactivity eluted with the same retention time as authentic [^{14}C]2105 at any sampling time.

Discussion

The radioactivity in the blood was located almost entirely in the plasma for 3 to 4 days after dosing. Only low levels of radiolabel were detected associated with the cellular components of the blood. The radioactivity in the plasma was associated with albumin and α_2-macroglobulin; only a trace of free [^{14}C]2105 was detectable in the plasma. Preliminary data suggest that ISIS 2105 binds to these proteins with a relatively low affinity and that binding is clearly saturable (Cossum et al., manuscript in preparation). The radioactivity bound to those plasma proteins represented mostly intact [^{14}C]2105. However, the levels of radioactivity in plasma were insufficient to determine the integrity of the drug at times beyond 8 hr postdosing.

There was a rapid and substantial distribution of radioactivity from the blood into the tissues. The initial volume of distribution of *22.0* ml approximates the blood volume of the rats used in this study. The postdistribution volume of distribution was 1076 ml, a value that indicated the distribution of radioactivity into a "deep" compartment. A four-compartment model fit the data best (*i.e.,* r^2 *0.998 vs. 0.823* for a two-compartment model). However, in other studies we have performed, a two-compartment model fit the data best. Consequently, we think the complexity of the model most likely results from minor animal-to-animal variations and the fact that radioequivalents were considered rather than intact drug. In future studies, we will attempt to address this issue in more detail. In any event, the elimination half-life was prolonged and examination of intact drug levels suggested a relatively prolonged elimination half-life for the intact drug and radioequivalents. The primary organs of accumulation of radioactivity were the liver, kidneys (particularly the renal cortex), bone marrow and spleen. The kinetics of distribution of the radiolabel into the peripheral organs varied. Peak levels were achieved in the liver and kidney 4 hr after the dose. By contrast, peak levels in the skeletal muscle were observed 1 hr after the dose and peak levels in the bone *marrow* were not achieved until 24 hr postdose. Skin accumulated a surprising amount of radiolabel, with peak levels that occurred 2 to 4 hours after administration.

That the radioactivity in various organs represented intact [^{14}C]2105 and metabolites was demonstrated by extraction followed by SAX-HPLC. Although its metabolism in the liver was extensive, the rate of metabolism was relatively slow. Twenty-four hours postdosing, approximately 15% of the total hepatic radioactivity was present as intact [^{14}C]2105. The metabolism in the kidney was minimum because intact drug was present even 96 hr postdosing. The extensive metabolism in the liver, coupled with the lack of metabolites found in the kidney and complete absence of intact drug in the urine, suggested that, after the initial distribution, only limited redistribution between the liver and kidney (and presumably other organs) occurred.

In this study, ^{14}C-labeled ISIS 2105 was synthesized using ^{14}C-labeled thymidine labeled at the carbon-2 position of thymine. The fate of thymidine is either through utilization into DNA *or* degradation to thymine (Henderson and Patterson, 1973). In mammals, the carbon-2 position carbon of thymine is degraded to CO_2 and so production of $^{14}CO_2$ would be expected when thymine is labeled at that carbon (Henderson and Patterson, 1973). Thymine could have been generated from ISIS 2105 in vivo in at least two ways. The putative metabolic scheme would involve hydrolysis of the phosphorothioate backbone, which eventually would generate thymidine. Subsequently, thymidine phosphorylase could dethiophosphorylate the thymi-

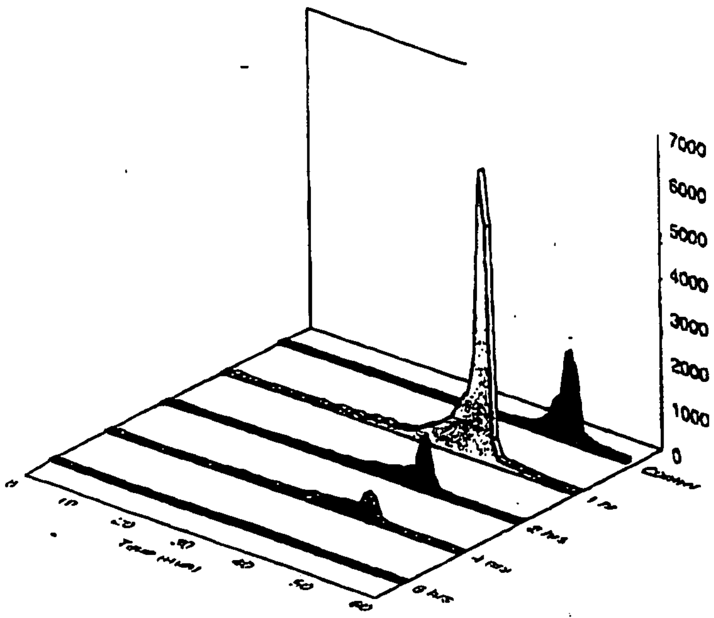

Fig. 4. SAX-HPLC radiochromatograms of plasma sampled from female Sprague-Dawley rats at various times after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg. The plasma contained radioactivity that eluted principally with [^{14}C]2105 (approximately 43 min).

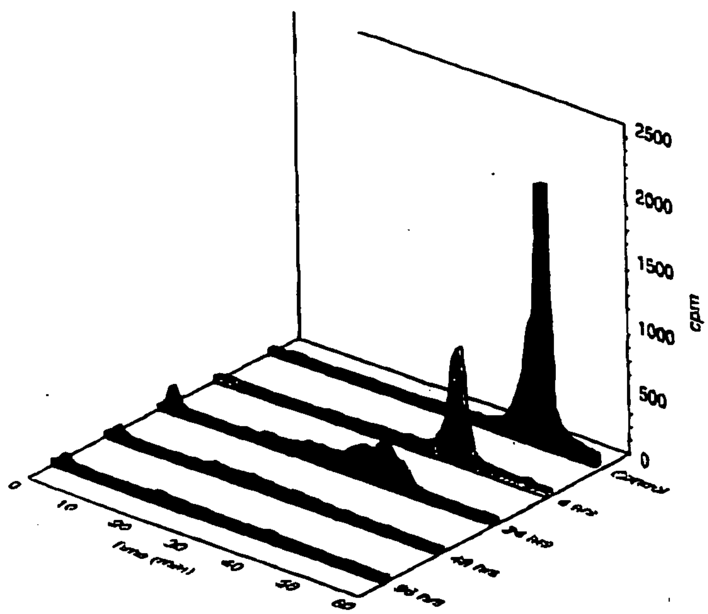

Fig. 5. SAX-HPLC radiochromatograms of extracts of liver sampled from female Sprague-Dawley rats at various times after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg. Authentic [^{14}C]2105 elutes at approximately 43 min.

dine or the thiophosphate might be oxidized first and the phosphorylase then would release phosphate. Thymine could then be further metabolized to CO_2. Thymidine phosphorylase is a cytoplasmic enzyme with highest activity in the intestinal mucosa, liver, bone marrow, kidney and spleen (Friedkin and Roberts, 1954). Consequently, the limited amounts of degradation products in the kidney suggest that the rate-limiting step in the pathway is hydrolysis. This is consistent also with the slow overall metabolism observed. In this regard, it is important also to recognize that approximately 2.4% of the internucleotide linkages in [^{14}C]2105 were phosphate resulting from the oxidation of the phosphorothioate during synthesis. This is greater than the routine specification for unlabeled ISIS 2105 (0.6%) and could account for a slightly greater hydrolytic rate of radiolabeled ISIS 2105.

Alternatively, it is possible that thymine was removed from [^{14}C]2105 by a glycosidic bond cleavage without prior hydrolysis of the internucleotide linkage. DNA glycosylases are nuclear enzymes that remove purines or pyrimidines from DNA as part of repair mechanisms. The resulting apurinic or apyrimidinic site in the oligonucleotide would be expected to be susceptible to endonuclease action (Warner, 1983). Further studies are required to elucidate the mechanism(s) of ISIS 2105 degradation.

The principal, albeit slow, mechanism of clearance of ISIS 2105 in rats is metabolism. Of the total dose, in excess of 50%

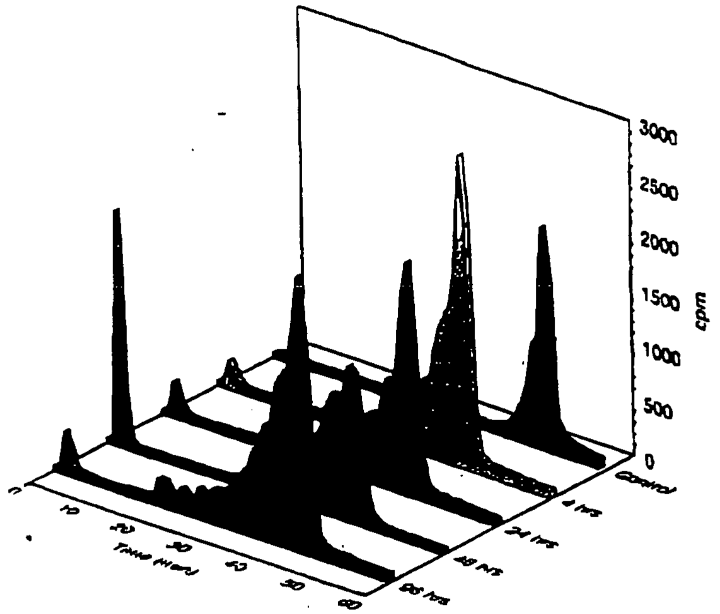

Fig. 6. SAX-HPLC radiochromatograms of extracts of renal cortex sampled from female Sprague-Dawley rats at various times after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg. Authentic [^{14}C]2105 elutes at approximately 43 min.

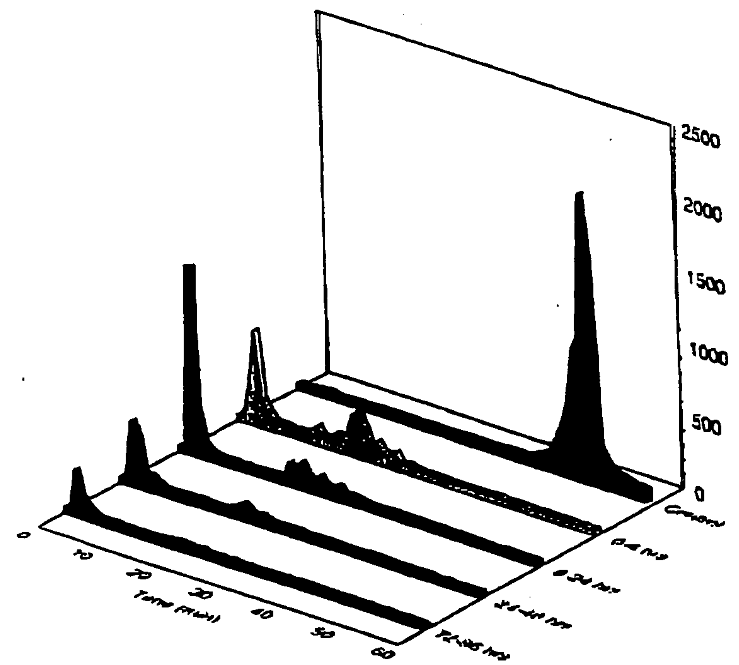

Fig. 7. SAX-HPLC radiochromatograms of urine sampled from female Sprague-Dawley rats at various times after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg. Authentic [^{14}C]2105 elutes at approximately 43 min.

was recovered in expired air. Only metabolites were found in the urine and urinary excretion accounted for only 15% of the total dose. There was no evidence of significant biliary secretion or hepatobiliary recirculation.

Although the results of this study and the study of Agrawal et al. (1991) were similar, there were several important differences. Agrawal et al. (1991) did not report on plasma protein binding. ISIS 2105, and every other phosphorothioate oligonucleotide we have studied, binds extensively to plasma proteins. The binding is low affinity and high capacity, which is traditionally associated with many other classes of drugs and their interaction with plasma proteins. We consider this to be one of the principal reasons that phosphorothioate oligonucleotides are not cleared rapidly by renal filtration. At the 30-mg/kg dose used by Agrawal et al. (1991), we would expect the plasma protein binding to be saturated and, therefore, to result in significant levels of free drug in the plasma. Approximately 30% of the i.v. or i.p. dose of radioactivity after dosing with the ^{35}S-labeled oligonucleotide was recovered in the urine by 24 hr postdosing and gel electrophoresis of the urine indicated the presence of intact drug. At early times (0–6 hr) after i.v. dosing, 95% of the urinary radioactivity coeluted with intact drug and,

up to 24 hr postdosing, only 15% degradation was noted. The urine of mice dosed i.p. contained material that was only 10% intact parent drug at 24 hr postdosing. At no time did we observe intact drug in the urine.

Two preliminary reports of studies on an anti-rev 27-mer phosphorothioate oligodeoxynucleotide have been presented (Bigelow et al., 1991; Bigelow et al., 1992). In these studies, the drug was given by a variety of routes (i.v. bolus, i.v. infusion, s.c. and p.o.) and concentrations of intact drug were determined by HPLC separation and ultraviolet detection. Excellent bioavailability from s.c. sites and limited p.o. bioavailability were reported. Tissue accumulation similar to our results was reported. A significantly shorter plasma half-life was reported but this was probably the result of the relatively insensitive detection methods. We have studied the pharmacokinetics of several oligonucleotides after intradermal, i.m., i.p. and intravitreal administration and articles describing these results are in preparation.

Agrawal et *al.* (1991) reported that 85% to 90% of the radioactivity present in most tissues of mice 48 hr after the administration of a ^{35}S-labeled phosphorothioate oligonucleotide was associated with intact drug. However, only 50% of the radioactivity in the liver and kidneys was associated with the parent drug at 48 hr. Only approximately 5% of hepatic radioactivity was present as apparently unchanged [^{14}C]2105. By contrast, the majority of renal cortex radioactivity in 96-hr samples eluted with authentic [^{14}C]2105. Larger molecular weight bands were found when tissue extracts were analyzed by polyacrylamide gel electrophoresis in the study of Agrawal et *al.* (1991). They speculated that the radioactivity might represent longer oligonucleotides. We did not observe any evidence of these species. An alternative explanation for these observations might be that the material represented drug bound to α_2-macroglobulin, a protein which is found in mice (LaMarre et *al.*, 1991).

All these differences might be explained by variations between species or differences caused by different sequences. Moreover, Iverson (1991) reported that, at lower doses, an anti-*rev* oligonucleotide was excreted intact in urine, which further suggests that there may be sequence differences. However, in studies in our laboratories, we have not observed such significant differences as a function of species or the specific sequence of the drug. Additional studies are clearly indicated.

In summary, after i.v. administration of [^{14}C]2105 to rats, the tissue distribution of radioactivity was extensive and the radioactivity was eliminated slowly. Although the drug was apparently extensively metabolized, the available evidence shows that the rate of metabolism was relatively slow. Metabolic studies were facilitated by synthesizing an oligonucleotide containing ^{14}C-labeled thymidine. Circulating radioactivity was extensively bound to plasma proteins, a phenomenon that may retard the renal filtration of unchanged drug. Finally, we observed binding of ISIS 2105 to α_2-macroglobulin. In addition to providing a plasma reservoir of ISIS 2105, biding to α_2-macroglobulin could be important in the pharmacokinetics of ISIS 2105 because this protein has been shown to be taken up by various cells through a receptor-mediated mechanism (James, 1990).

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Blocking of Heart Allograft Rejection by Intercellular Adhesion Molecule-I Antisense Oligonucleotides Alone or in Combination with Other Immunosuppressive Modalities[1]

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Intercellular adhesion molecule-I (ICAM-1) binds circulating leukocytes through interactions with β_2 integrins, LFA-1, and macrophage Ag-1. The phosphorothioate antisense oligodeoxynucleotide, IP-3082, specific for ICAM-1mRNA inhibited ICAM-1, but not vascular cell adhesion molecule-I, mRNA induction and expression of ICAM-1 molecules by mouse endothelioma cells. Scrambled control oligonucleotides were ineffective. Untreated C3H (H-2^k) mice rejected C57BL/10 (H-2^b) heart allografts with a mean survival time of 7.7 ± 1.4 days. Administration Lv. of IP-3082 by a 7-day osmotic pump prolonged the survival of heart allografts in a dose-dependent fashion: 1.25 mg/kg, to 11 ± O.days; 2.5 mg/kg, to 12 ± 2.7 days; 5 mg/kg, to 14.1 ± 2.7 days; and 10 mg/kg, to 15.3 ± 5.8 days (all $p < 0.01$). Control IP-1082 (10 mg/kg) was ineffective (7 ± 0.8 days). Although 7-day anti-LFA-1 mAb (SO &day; i.p.) prolonged allograft survival to 14.1 ± 2.7 days, the addition of IP-3082 (5.0 mg/kg x 7 days) induced donor-specific transplantation tolerance (>150 days). Furthermore, IP-3082 (5 mg/kg X 7 days) acted synergistically with antilymphocyte serum, rapamycin, and brequinar, but not cyclosporin A: a single antilymphocyte serum (0.2 ml) i.p. injection alone prolonged graft survival to 10 ± 0.5 days ($p < 0.01$) and in combination with IP-3082 (5 mg/kg), to 32.2 ± 8.3 days ($p < 0.001$); rapamycin (0.1 mg/kg X 7 days; i.v.) alone prolonged survival to 13 ± 7.5 days ($p < 0.01$), and with IP-3082, to 32.4 ± 8.9 days ($p < 0.03$); brequinar (0.5 mg/kg X 7 days; oral gavage) alone to 12 ± 2.4 days ($p < 0.05$), and with IP-3082 (5 mg/kg), to 38.8 ± 30.2 days ($p < 0.01$); in contrast, cyclosporin A (5 mg/kg X 7 days; i.v.) alone produced graft survival of 9.8 ± 1.3 days ($p <$ 0.1 and in combination with IP-3082 (5 mg/kg), produced survival of 7.8 ± 3.5 days (NS). Thus, antisense oligonucleotides may proffer a selective gene-targeted immunosuppressive therapy for organ transplantation. *The Journal of Immunology,* 1994, 153: 5336.

Numerous studies have demonstrated the inhibition of viral and cellular gene expression after treatment of cells in culture with oligonucleotides designed to hybridize to a specific mRNA by Watson-Crick base pairing (I-3). Antisense oligonucleotides may inhibit protein expression by multiple mechanisms, including translational arrest, inhibition of RNA processing, and promotion of the degradation of the targeted RNA. Although unmodified phosphodiester oligonucleotides are effective in in vitro assays, their rapid hydrolysis by serum and cellular nucleases limits their utility in vivo (4, 5). In contrast, phosphorothioate oligodeoxynucleotides (PS-oligos),[3] in which sulfur is substituted for one of the nonbridging oxygen atoms in the phosphate backbone, demonstrate greatly increased stability in relation to serum and cellular nucleases and, therefore, may be very useful for in vivo applications (6).

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[3] Abbreviations used in this paper: PS-oligo, phosphorothioate oligonucleotides; ALS, antilymphocyte serum; BQR, brequinar; CsA, cyclosporin A; DOTMA/DOPE, N-[1(-2,3(-dioleyloxy)propyl]]-N,N,N-trimethylammonium chloride/dioleoylphosphatidylethanolamine; ICAM-1, intercellular adhesion molecule-I; MAC-1, macrophage Ag-1; RAPA, rapamycin; VCAM-1, vascular cell adhesion molecule-I; VLA-4, very late Ag-4; MST, mean survival time; CI, combination index; q.o.d., every second day; G3PDH, glyceraldehyde triphosphate dehydrogenase.

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As was recently demonstrated, PS-oligos designed to hybridize to different regions of human intercellular adhesion molecule-1 (ICAM-1; CD54) mRNA inhibited ICAM-I expression in a sequence-specific manner (7). The most active PS-oligo hybridized to sequences located in the 3'-untranslated region of mRNA. The ICAM-1 molecule belongs to the Ig-related cell adhesion molecule family and is expressed at a low level on the surface of endothelial cells and leukocytes (8-10). In response to pro-inflammatory cytokines, such as IL-l, TNF, and IFN-γ, the expression of ICAM-1 molecules is markedly increased on a variety of cells, including endothelial cells and fibroblasts, and is induced on keratinocytes, smooth muscle cells, and cardiac myocytes (11). ICAM-1-expressing endothelial cells bind circulating leukocytes through interactions with the β_2 integrins, namely LFA-1 (CD1 1 a) and MAC-l, thereby facilitating emigration of leukocytes out of the vasculature (12, 13).

Furthermore, ICAM-1 interaction with LFA-1 has been shown to enhance T cell interaction with APC, T cell-dependent activation of B cells, and the killing of target cells by neutrophils, NK cells, or Ag-specific cytotoxic T cells (14-18). Adhesion of leukocytes to endothelial cells requires three overlapping steps, namely rolling, chemoattraction, and strong adhesion (19, 20). Upon activation by pro-inflammatory mediators, a family of selectins, namely granule-external membrane protein (P-selectin; CD62P), endothelial-leukocyte adhesion molecule (E-selectin; CD62E), and lymph node homing receptor (L-selectin; bind to the carbohydrate ligands (sialylated Lewis" and Lewis"), thereby triggering an initial tethering of flowing leukocytes to the vessel wall (10, 21). The rolling of leukocytes along the surface of the endothelial cells enhances their exposure to chemoattractants that transduce signals, thereby increasing the avidity of integrins (22). The firm attachment of leukocytes to endothelial cells involves an interaction between integrins (LFA-1, MAC-l, and very late Ag-4 (VLA-4) molecules) on circulating leukocytes and Ig-related cell adhesion molecules (ICAM-1, ICAM-2, ICAM-3, and vascular cell adhesion molecule- 1 (VCAM-1)) on the endothelial cells (23). Increased expression of ICAM-1 molecules correlates with increased leukocyte infiltration followed by the rejection of organ allografts in both humans and mice (24-26). Direct evidence for the involvement of ICAM-1 and LFA-1 molecules in allograft rejection was provided by the treatment of heart allograft recipients with anti-ICAM- 1 (YN l/l .7.4) and anti-LFA-1 (KBA) mAb (27). A 7-day i.p. therapy with anti-ICAM- 1 or anti-LFA- 1 mAb prolonged the survival of heart allografts. However, the combination of anti-ICAM- 1 and anti-LFA- I mAb induced donor-specific transplantation tolerance. These findings suggest that reduction in the expression of ICAM-1 followed by decreased appearance of LFA-1 -expressing cells may produce potent in vivo immunosuppressive activity. We found that mouse ICAM-1 antisense PS-oligos inhibited the rejection of heterotopic cardiac allografts and, in combination with anti-LFA-1 mAb, induced donor-specific transplantation tolerance. Furthermore, ICAM-1 antisense PS-oligos combined with antilymphocyte serum (ALS), rapamycin (RAPA), or brequinar (BQR), but not with clospdrin A (CsA), synergistically inhibited the rejection of heart allografts.

Materials and Methods

Cells and reagents

Fetal bovine serum was purchased from HyClone (Logan, UT). DMEM and Dulbecco's PBS were purchased from Irvine Scientific (Irvine, CA). Opti-MEM serum-free medium was obtained from Life Technologies (Grand Island, NY). Primaria 96-well plates were obtained from Falcon Labware (Lincoln Park, NJ). Human rTNF-α and mouse IFN-γ were purchased from R&D systems (Minneapolis, MN). Anti-LFA-I mAb were obtained from Dr. Yogita (Juntendo University School of Medicine, Tokyo, Japan), and anti-VCAM-1 mAb were purchased from Genzyme (Cambridge, MA). Anti-ICAM-I mAb (YN1/1.7.4; American Type Culture Collection, Rockville, MD) were purified from ascites on a protein G column. DOTMA/DOPE (*N*-[1(-2,3-dioleyloxy)propyl]]-*N*,*N*,*N*-trimethyl-ammonium phosphatidylethanolamine) solution (lipofectin), biotinylated goat anti-mouse IgG, and β-galactosidase-conjugated streptavidin were purchased from Bethesda Research Labs (Bethesda, MD). The DNA synthesizer reagents, controlled pore glass-bound and β-cyanoethyldiisopropylphosphoramidites, were obtained from Applied Biosystems (Foster City, CA). Centrex filters were purchased from Schleicher & Schuell (Keene, NH). Zeta-Probe nylon blotting membranes were purchased from Bio-Rad (Richmond, CA). QuickHyb solution was purchased from Stratagene (La Jolla, CA), and the cDNA labeling kit, Prime-a-Gene, was obtained from Promega (Madison, WI).

PS-oligo synthesis

PS-oligos were synthesized on a OS-mmole scale on a Milligen model 8800 DNA synthesizer by using modified phosphoramidite chemistries with β-cyanoethoxyphosphoramidites (7). A crude product of approximately 70% purity was further purified by orthagonal column chromatography by using a Millipore HCl 8-HA column followed by anion exchange chromatography on a Millipore Q-15 strong anion exchanger. The purified material was ethanol precipitated, redissolved, and further desalted by ultrafiltration. The samples were depyrogenated by ultrafiltration with endotoxin levels that had been reduced to below detectable levels. The sequences of the PS-oligos were: IP-3068, AGCTGCGCTGCTACCTGCAC; IP-3069, GCCCATTGCAGGGCCAGGGC; IP-3066, GGGTTGAAGCC ATTGCAGGG; IP-3070, CTCATCCAGCAGGCTCAGGG; IP-3065, CCAGAGGAAGTGGCTGAGGG, IP-3082, TGCATCCCCCAGGCCAC CAT; IP-3806, CAAGTGTGCATCCCCCAGGC; IP-3083, TTGGGAC AATGTCTCAGCTT, IP-3084, TGCCAGTCCACATAGTGTTT; and IP-3099, TGCTTACCCTCCCACAGCAG. The control PS-oligo sequences were: IP-3823, TGCCCCTCACCAGCACGCAT, IP-8997, TCGCATC GACCCGCCCACTA, and IP-4 1 89, CAGCCATGGTTCCCCCCAAC, which were scrambled IP3082 sequences; and IP-1082, GCCGAGGTC CATGTCGTACGC, which targets the herpes virus UL- 13 gene sequence.

PS-oligo treatment

The bEND.3 cells were kindly provided by Dr. Werner Risau, Max-Planck Institute, Planegg-Martinsreid, Germany. Cells were treated with indicated concentration of PS-oligo in the presence of 15 μg/ml DOTMA/DOPE liposome formulation for 4 h. ICAM-I expression was induced by treatment with 5 ng/ml human rTNF-α and 1000 U/ml murine IFN-γ for 16 h (7). Cells were fixed with 95% ethanol, and ICAM-I expression was quantified by incubation with ICAM-I mAb (YN1/1.7.4), followed by incubation with a biotinylated goat anti-rat IgG Ab and streptavidin conjugated β-galactosidase. Results are expressed as the percentage of control ICAM-I expression, which was calculated as follows: [((ICAM-I expression for oligonucleotide-treated cytokine-induced cells) − (basal ICAM-I expression))/((cytokine-induced ICAM-I expression) − (basal ICAM-I expression))] X 100. Both basal and cytokine-treated cells were pretreated with DOTMA.

RNA isolation and analysis

Total cellular RNA was isolated by cellular lysis in 4 M guanidinium isothiocynate followed by a cesium chloride gradient (28). Total RNA was separated on a 1% agarose gel containing I. 1% formaldehyde and transferred to nylon membranes. Blots were hybridized for I to 2 h in QuickHyb solution with the ICAM-I cDNA probe. which had been labeled with [^{32}P]dCTP with use of random oligonucleotide primers. Blots were washed with 2X SSC containing 0.1% SDS at 25°C then washed in 0. IX SSC containing 0. I% SDS at 60°C. Quantitation of RNA expression was performed by using a PhosphorImager (Molecular Dynamics, Sunnyvale. CA).

Mice

C3H (H-2^k), C57BL/10 (H-2^b). C57BL/6 (H-2^b). and BALB/c (H-2^d) female mice. 4 to 6 wk old, were purchased from Harlan Sprague-Dawley (Indianapolis. IN). housed in cages in room with controlled light/dark cycles, and supplied food and water ad libitum. All experiments were approved by the Animal Welfare Committee and conducted in accordance with the University of Texas Guidelines.

Heart transplantation

Heterotopic heart transplantation was performed according to the previously described method (29). Donor hearts were perfused through the vena cava and aorta with cold heparinized saline before harvesting and ligation of the vena cava and pulmonary veins. The donor pulmonary artery was anastomosed to the vena cava of the recipient, and the donor aorta was anastomosed to the recipient aorta, by using I I-O nylon suture (Ethicom Inc.. Sommerville. NY). Cold ischemia time was less than 30 min. Heart allograft function was examined by daily palpation, and the day of heartbeat cessation was regarded as the day of rejection.

Immunosuppressive modalities

CsA (Sandoz. Basel. Switzerland) dissolved in cremophor (Sigma Chemical Co.. St. Louis, MO) was delivered via jugular venous infusion by a 7day osmotic pump (Alzet, Palo Alto, CA). RAPA (Wyeth Ayerst, Rouses Point, NY) in a dilutant (Tween 80, 10%; *N-N*-dimethylacetamide, 20%; and polyethylene glycol 400. 70%) was infused i.v. by 7-day osmotic pump. BQR (DuPont, Wilmington, DE) diluted in distilled water was administered every second day (q.o.d.) by oral gavage for 7 days. Rabbit anti-mouse ALS (Accurate Chemicals. New York, NY) was injected once i.p. two days before grafting.

Statistical analysis

Heart allograft survivals are presented as a mean survival time (MST ± SD), with comparison among groups being performed by Gehan's survival test. The median-effect principle (30, 31) is on the basis of the premise that the effect of each agent is related to its dose and, therefore, may be calculated by using the following equation:

$$\left(\frac{fa}{fu}\right) = \left(\frac{D}{Dm}\right)^m$$

Where fa and fu represent the fractions of the system that are affected (percent inhibition or days of survival beyond controls) and unaffected (I-fa), respectively. by the drug at dose D. Full protection (fa = 1) is defined as at least a 50day survival of heart allografts. *Dm* is the dose required for 50% inhibition (ED$_{50}$), the median effect; *m* is a coefficient that describes the sigmoidicity of the dose-effect curve. Logarithmic conversion of the median-effect equation linearizes the relationship:

$$\log\left(\frac{fa}{fu}\right) = m \log (D) - m \log (Dm)$$

This relation defines *m* as the slope of the plot log (fa/fu) vs log dose and log *Dm* as the X-intercept when log (fa/fu) = 0 or fa = fu = 0.5. The *m* value describes the shape of the dose-effect curve. which is hyperbolic when *m* equals I and sigmoidal or negatively sigmoidal when *m* is higher or lower than I. respectively. The linear regression coefficient *r* describes the "goodness" of the fit of the data to the median-effect principle. A minimum, *r* of 0.75 was required to conclude that the data obey the median-effect principle. The interaction between two drugs was assessed by using the combination index (CI) method (31) for the doses to achieve *x*% inhibition (days of graft survival):

$$CIX = \frac{D_1 \text{ combined}}{(Dx)_1 \text{ alone}} + \frac{D_2 \text{ combined}}{(Dx)_2 \text{ alone}}$$

for the mutually exclusive case, where both drugs have the same or similar modes of action, or the more conservative expression:

$$CIX = \frac{D_1 \text{ combined}}{(Dx)_1 \text{ alone}} + \frac{D_1 \text{ combined}}{(Dx)_2 \text{ alone}} + \frac{(D_1 \text{ combined})\ (D_2 \text{ combined})}{[(Dx)_1 \text{ alone}]\ [(Dx)_2 \text{ alone}]}$$

for the mutually nonexclusive case, where each drug has a different mode of action. Computer software (32) was used to automatically determine the dose-effect parameters (*Dm*, *m*, and *r*) and the CI values.

Results

Selection of ICAM-1 antisense PS-oligos by in vitro analysis

Comparison of the nucleotide sequences of human and murine ICAM-1 cDNAs revealed that the cDNA sequences are conserved only 65% (7, 33). The degree of conservation was even lower within the untranslated 3'-regions, where the most active human antisense PS-oligos were identified. Therefore, it was necessary to identify PS-oligos that selectively target murine ICAM-1 mRNA. To select the most effective PS-oligos for in vivo studies, ten PS-oligos (20 mer) were designed to hybridize to different regions on the mouse ICAM-1 mRNA (Fig. 1A). In particular, we selected three mouse sequences in the untranslated 5'-region and seven mouse sequences in the untranslated 3'-region. The PS-oligos were screened by an ELISA in the presence of a cationic liposome formulation (DOTMA/DOPE) for inhibition of ICAM-1 induction in a murine endothelioma cell line, bEND.3. The bEND.3 cells expressed a basal level of ICAM-1 that increased significantly after treatment with a combination of human TNF-α and murine INF-γ. A 4-h preincubation with the PS-oligo, followed by a 16-h culture with cytokines, produced different effects on ICAM-1 expression. Although all of the ICAM-1 antisense PS-oligos inhibited cytokine-induced ICAM-1 expression (Fig. 1*B*), IP-3082 and IP-3806, which hybridized to the 3'-untranslated region of the ICAM-1 mRNA, were able to lower ICAM-1 protein expression to below the basal level of expression (Fig. 1*B*). IP-3082 was chosen for further evaluation by using an in vitro cellular assay to measure ICAM-1 and VCAM-1 mRNA expression (Fig. 2). Treatment of bEND.3 cells with 50 nM IP-3082 selectively reduced cytokine-induced ICAM-1 mRNA by 90% (Fig. 2A). The effect of IP-3082 on ICAM-1 mRNA was sequence specific; three scrambled (IP-3823, IP-8997, and IP-4189) and one unrelated (IP-1082) PS-oligos failed to affect ICAM-1 mRNA expression. In fact, two scrambled PS-oligos, IP-8997 and IP-4189, slightly enhanced ICAM-1 mRNA expression. The effect on ICAM-1 was selective: IP-3082 and two controls (IP-3823 and IP-8997) only slightly reduced

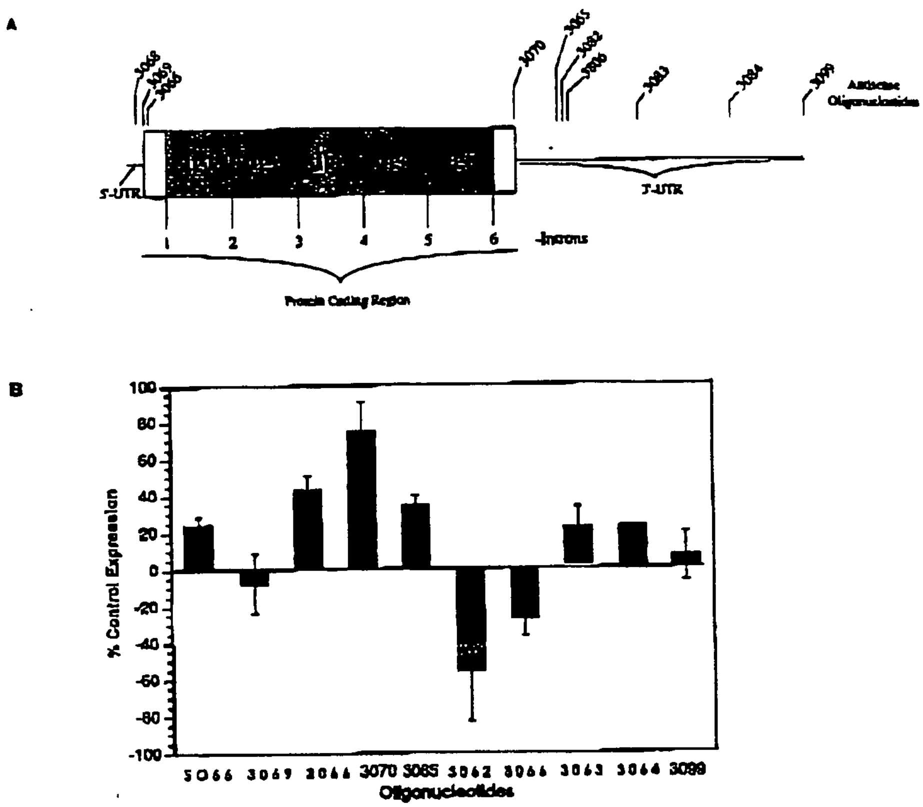

FIGURE 1. **Identification of a murine ICAM-1 antisense PS-oligo.** *A)* **the approximate position of 10 PS-oligos designed to hybridize to different regions of the ICAM-1mRNA:** I-3068, I-3069, and I-3066 in the untranslated 5'-end region; and I-3083 **and I-3084 in the untranslated 3'-end regions.** *B)* **murine endothelioma cells, bEND.3, were treated with 300 nM of the** indicated PS-oligo in the presence of 15 μg/ml DOTMA/DOPE liposome formulation for 4 h in serum-free medium, as previously described by Chiang et al. (7) and Bennett et al. (57). Medium was replaced with DMEM containing 10% fetal bovine serum, and ICAM-1 expression was induced with human TNF-a (5 ng/ml) and murine IFN-γ (1000 U/ml). ICAM-1 expression was quantified 16 h after induction by an ELISA (7). Under basal **conditions, approximately 25% of the bEND.3 cells express** ICAM-1 as determined **by flow cytometry (data not shown).**

VCAM-I mRNA expression (up to 25%). Furthermore, IP-3082 and control PS-oligos did not affect the glyceraldehyde triphosphate dehydrogenase (G3PDH) transcripts (Fig. 2C). These results showed that, among 10 ICAM-1 antisense PS-oligos, IP-3082, which hybridizes to the 3'-end untranslated region, effectively inhibited ICAM-I protein expression and reduced the level of ICAM-1mRNA in bEND.3 endothelial cells. Thus, in vitro data strongly suggest that IP-3082 inhibits ICAM-I expression by means of an antisense mechanism.

Effect of ICAM- 7 antisense PS-3082 on heart allograft survival

The in vivo activity of PS-oligos was examined in a mouse heterotopic cardiac transplant model (29). ICAM- I antisense PS-oligo, IP-3082, unrelated IP- 1082 control, or scrambled IP-4 189 control was delivered i.v. by a 7-day osmotic pump. Untreated C3H(H-2^k) mice rejected C57BL/10 (H-2') vascularized heart allografts at a MST of 7.7 ± 1.4 days (Table I). A 7-day infusion of the control IP-1082 at a dose of either 5 or 10 mg/kg/day did not affect allograft survival. Similarly, a scrambled IP-4189 control (10 mg/kg) did not significantly affect heart allograft survival (9 ± 0.8 days; NS). In contrast, infusion of the ICAM-I antisense IP-3082 prolonged allograft survival in a dose-dependent fashion: 1.25 mg/kg/day of IP-3082 prolonged graft survival to 1 1± 0 days; 2.5 mg/kg/day, to 12 ± 2.7 days; 5 mg/kg/day, to 14.1 ± 2.7 days; and 10 mg/kg/day, to 15.3 ± 5.8 days (all $p < 0.01$). Extended 14-day treatment with IP-3082 (5 mg/kg/day) further increased graft survival up to 30 **days.** Almost identical results were produced in two additional donor-recipient combinations, namely BALB/c (H-2^d) to C3H and C57BL/6 (H-2^b) to BALB/c (data not shown). The effectiveness of the immunosuppression was documented by histologic examination of grafts on day 6 after transplantation. Syngeneic C57BL/1 0 hearts transplanted to C57BL/10

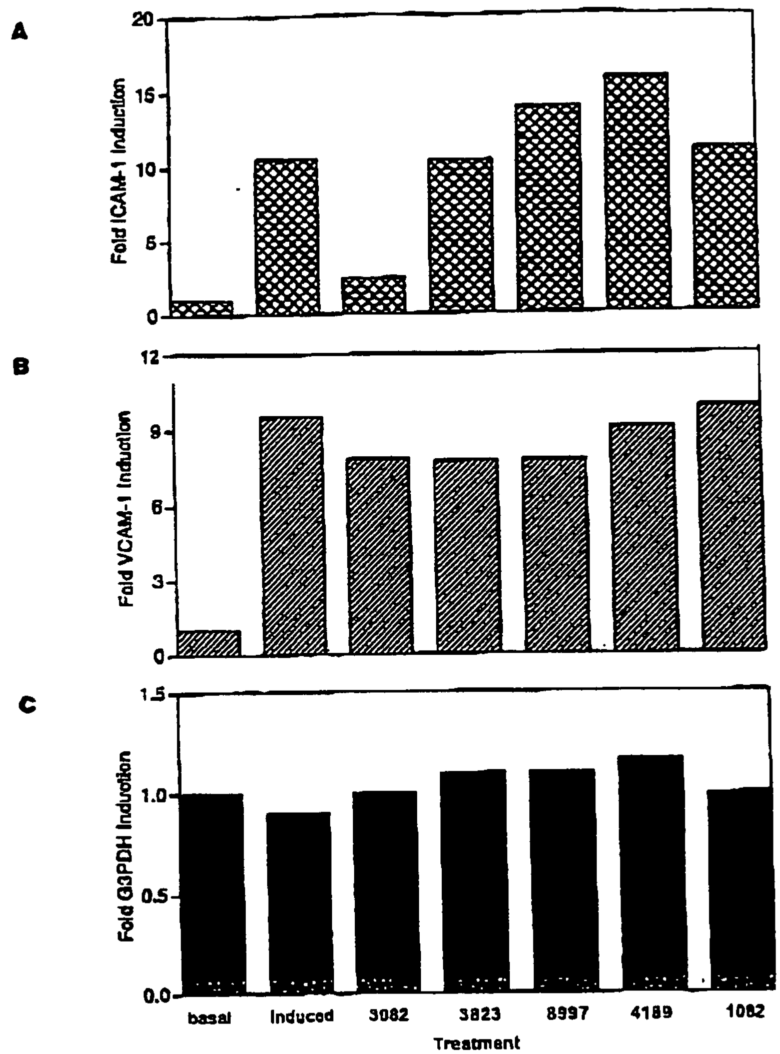

FIGURE 2. Histology of C57BL/10 hearts. *A*) normal, control heart. *B*) syngeneic heart graft 6 days after transplantation demonstrating mild infiltration of 10% of the tissue. *C*) allogeneic heart graft 6 days after grafting to untreated recipient demonstrating massive infiltration of leukocytes and neutrophils with severe necrosis and interstitial hemorrhage. *D*) allogeneic graft 6 days after transplantation to recipient treated with 10 mg/kg/day ICAM-1 I-3082 demonstrating mild infiltration of 20% of the tissue. All sections were fixed in 10% formalin and stained with H&E. Original magnification is X200.

Table I. *Effect of ICAM-1 antisense IP-3082 on C57BL/10 (H-2[b]) heart allograft survival in C3H (H-2k) recipients*[a]

Treatment				
PS-oligos (days)	mg/kg/day	Graft Survival (days)	MST ± SD	p
None	–	6, 7 x 3, 8, 9, 10	7.7 ± 1.4	–
IP-1082 (×7)	5.0	6, 7 × 3, 8 × 2	7.1 ± 0.7	NS
	10.0	6, 7 X 2, 8	7.0 ± 0.8	NS
IP-4189 (×7)	10.0	8, 9 × 2, 10	9.0 ± 0.8	NS
IP-3082 (×7)	1.25	11 x3	11.0 ± 0	0.001
	2.5	9, 10, 12, 13, 16	12.0 ± 2.7	0.01
	5.0	10, 12 X 2, 13, 16 × 2, 17 × 2	14.1 ± 2.7	0.01
	10.0	12 X 2, 13, 24	15.3 ± 5.8	0.01
IP-3082 (X14)	5.0	16, 17, 29, 30	23.0 ± 7.5	0.001

[a] Recipients were untreated or infused i.v. by 7day (X7) osmotic pump with 5 or 10 mg/kg control IP-1082 (17), 10 mg/kg control IP-4189, or 1.25, 2.5, 5, or 10 mg/kg antisense IP-3082 starting at the day of transplantation. In one group of mice, 5 mg/kg IP-3082 was infused i.v. for 14 (× 14) days. Heart allografts were evaluated daily by palpation, and the day of heart beat cessation was considered the day of rejection.

mononuclear cells and neutrophils. This effect was associated with severe necrosis and mineralization, which formed a dense band that affected 60% of the epicardium, myocardium, and papillary muscles (Fig. 3C). In contrast, heart allografts from recipients treated with IP-3082 (5 mg/kg/day) showed only scattered infiltration with mononuclear

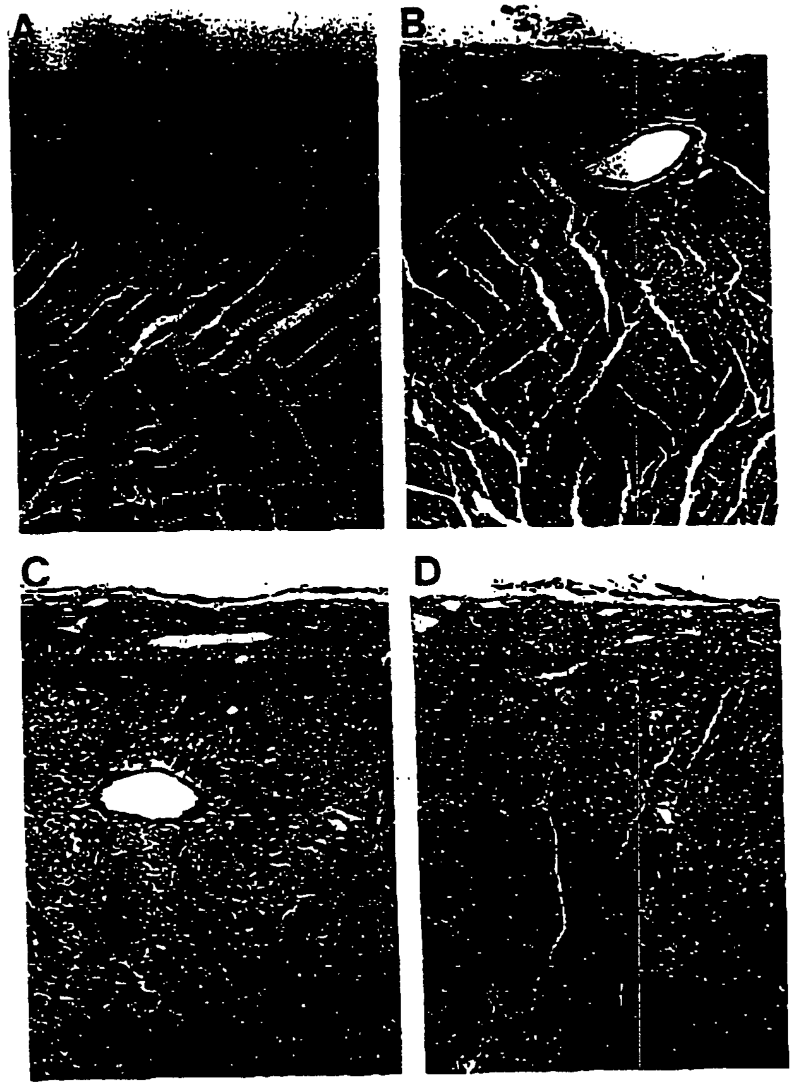

FIGURE 3. Histology of C57BL/10 hearts. A) normal, control heart. B) syngeneic heart graft 6 days after transplantation demonstrating mild infiltration of 10% of the tissue. C) allogeneic heart graft 6 days after grafting to untreated recipient demonstrating massive infiltration of leukocytes and neutrophils with severe necrosis and interstitial hemorrhage. D) allogeneic graft 6 days after transplantation to recipient treated with 10 mg/kg/day ICAM-1 I-3082 demonstrating mild infiltration of 20% of the tissue. All sections were fixed in 10% formalin and stained with H&E. Original magnification is X200.

formed a dense band that affected 60% of the epicardium, myocardium, and papillary muscles (Fig. 3C). In contrast, heart allografts from recipients treated with IP-3082 (5 mg/kg/day) showed only scattered infiltration with mononuclear cells in 20% of the myocardium (Fig. 30). Thus, we conclude that ICAM-I antisense IP-3082 inhibits infiltration and subsequent destruction of heart allograft tissue by host cells.

Combined effect of ICAM-1 antisense IP-3082 and anti-LFA-7 mAb on heart allograft survival

The in vivo effects of the ICAM-I antisense PS-oligo IP-3082 in conjunction with anti-LFA-I mAb (KBA) were examined in the C57BL/10 to C3H combination. A 7-day treatment with i.p. injections of anti-LFA-I mAb (50 μg/day) or i.v. infusion of IP-3082 (5 mg/kg/day) prolonged allograft survival to 10.6 ± 4.6 days and 14.1 ± 2.7 days, respectively (Fig. 4). Combined treatment with the two agents for 7 days resulted in indefinite survival of heart allografts (>150 days; $p < 0.001$). The interaction of IP-3082 and anti-LFA-I mAb was evaluated by the median effect analysis (30). The MST values revealed a dose-dependent effect of individual agents: linear regression coefficient (r) values were higher than 0.75, thereby allowing further analysis. The r value for IP-3082 was calculated on the basis of the results presented in Table I. Three individual doses of anti-LFA mAb were examined: 25 μg/day for 7 days was ineffective (7.7 ± 0.6); 50 μg/day prolonged graft survival to 10.6 ± 4.6 days; and 100 μg/day prolonged survival to 15.0 ± 5.9 days ($p < 0.01$). CI value calculated for a combination of 5 mg/kg/day IP-3082 and 50 μg/day anti-LFA- 1 mAb was 0.00 I, indicating strong synergism (CI = 1 shows additive, and CI <1 or CI >1 shows synergistic or antagonistic interaction, respectively). Recipients bearing C57BL/10 hearts for 65 days

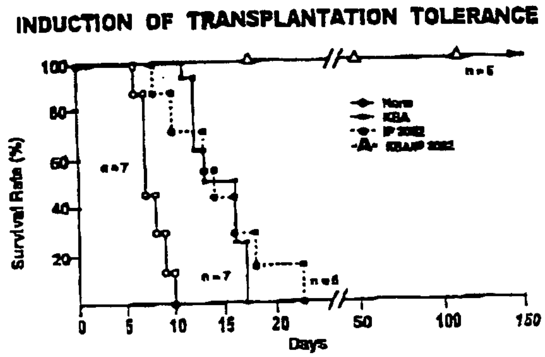

FIGURE 4. Combined effect of ICAM-1 antisense I-3082 and anti-LFA-1 mAb (KBA) on heart allograft survival. C3H recipients of C57BL/10 hearts were either untreated or treated with daily i.p. injection for 7 days of KBA Ab (50 μg/day) alone, I-3082 (5 mg/kg/day) i.v. by 7-day osmotic pump alone, or a combination of KBA mAb (50 &day) i.p. and I-3082 (5 mg/kg/day) i.v. for 7 days.

(n = 4) were transplanted with donor-type C57BL/10 and third-party BALB/c (H-2^d) skin allografts. The induction of transplantation tolerance was confirmed by permanent acceptance of donor-type skin grafts (> 100 days) and acute rejection of third party grafts in 9 ± 0 days (data not shown). Control C3H mice (n = 5) rejected C57BL/10 and BALB/c grafts in 9.2 ± 0.8 days and 8.1 ± 0.6 days, respectively. These results indicate that the combination of ICAM-1 antisense PS-oligo, PS-3082, and anti-LFA-1 mAb induces donor-specific transplantation tolerance.

Effect of ICAM-1 antisense IP-3082 in combination with ALS, RAPA, BQR, or CsA on heart allograft survival

The interaction of IP-3082 with different immunosuppressive agents, namely ALS (34), RAPA (35, 36), BQR (37), and CsA (38–40), was examined in C3H recipients of C57BL/10 heart allografts. Median effect analysis was used to calculate the individual effects of IP-3082 (Table I), ALS (Table II), RAPA (Table III), BQR (Table IV), and CsA (Table V) as well as the combined effects of IP-3082 with ALS, RAPA, BQR, or CsA. A single i.p. injection of ALS two days before transplantation prolonged graft survival: 0.1 ml prolonged graft survival to 9 ± 0 days; 0.2 ml, to 10.4 ± 0.5 days; and 0.4 ml, to 14 ± 2.1 days (all $p < 0.01$; Table II). The combination of 0.2 ml ALS and IP-3082 (5, 10, or 20 mg/kg/day X 7 days) extended allograft survivals to 32.2 ± 8.3 days, 37.0 ± 5.8 days, and 72.0 ± 49.1 days, respectively (all $p < 0.01$; CI < 0.001). RAPA (0.05, 0.1, or 0.2 mg/kg/day) alone delivered i.v. by a 7-day osmotic pump immunosuppressed rejection in a dose-dependent fashion (Table III). Combining 0.1 mg/kg RAPA with 5 mg/kg IP-3082 prolonged graft survival to 32.4 ± 8.9 days, and combining it with 10.0 mg/kg IP-3082, to 36.3 ± 6.1 days (both $p <$ 0.01; CI < 0.02). Oral gavage with BQR (0.5, 1, and 2 mg/kg/day) delivered every second day (q.o.d.) for 7 days prolonged allograft survival to 12 ± 2.4 days, 17.6 ± 3.4 days, and 20 ± 4.1 days, respectively (Table IV; all <0.01): the combination of 0.5 mg/kg BQR and 5 mg/kg IP-3082 resulted in a MST of 38.8 ± 30.2 days ($p < 0.01$; CI = 0.007). Although a 7-day i.v. infusion of 2.5 or 5 mg/kg/day CsA was ineffective, 10 or 20 mg/kg/day CsA prolonged allograft survival. Adding IP-3082 (5 or 10 mg/kg/day) to 5 mg/kg/day CsA did not improve graft survivals with CI values of 14.1 and 51, respectively (Table V). Similarly, combining control IP-1082 (5 mg/kg) and CsA (5 mg/kg) did not affect heart allograft survival (9.2 ± 2.2 days; NS: data not shown). These results show that ICAM-1 antisense IP-3082 synergistically interacts with different immunosuppressive modalities (ALS, RAPA, and BQR), but not with CsA, to block allograft rejection.

Discussion

These experiments were designed to select an ICAM-1 antisense PS-oligo for gene-targeted immunosuppressive therapy to block heart allograft rejection. The results documented that in vitro screening allowed the determination of a very effective target region for PS-oligo on mouse ICAM-1 mRNA. Interestingly, the most active PS-oligo (IP-3082) hybridized to the 3'-untranslated region of ICAM-1 mRNA. IP-3082 inhibited in vitro expression of cytokine-induced ICAM-1 protein on bEND.3 endothelioma cells. Furthermore, under the same experimental conditions, IP-3082 inhibited cytokine-induced ICAM-1 mRNA by 95%. Similar results were observed during analysis of 35 PS-oligos that target human ICAM-1 mRNA, which were the most effective PS-oligos hybridized to the 3'-untranslated region (7, 41). The human ICAM-1 antisense PS-oligos seem to inhibit ICAM-1 expression by a mechanism involving RNase H hydrolysis of the target mRNA. The inhibitory activity was lost when the ICAM-1 antisense PS-oligos were modified by substitution on the 2'-position of the sugar by fluoro, O-methyl, or *O*-propyl (7, 41). Such substitutions enhanced binding of the PS-oligo to the target mRNA, but do not support RNase H hydrolysis. Similar results were obtained with mouse antisense PS-oligos (data not shown), which suggests that PS-3082 inhibits ICAM-1 expression, at least in part, through a RNase H mechanism.

The in vitro studies were extended to an in vivo analysis to examine the effect of the ICAM-1 antisense PS-oligo, IP-3082, in a model of complex inflammatory response to organ allografts. Previously, it has been demonstrated that anti-ICAM-1 mAb significantly prolongs heart allograft survival, implicating ICAM-1 in the rejection process (27). The ICAM-1 antisense PS-oligo, IP-3082, extended heart allograft survival in a dose-dependent fashion when

Table II. *Combined effect of ALS and ICAM-1 antisense IP-3082 on heart allograft survival*[a]

Treatment					
IP-3082 ALS (ml)	(mg/kg/day)	Heart Survival (days)	MST ± SD	*P*	*CI*
None	–	6,7x3,8,9,10	7.7 ± 1.4	–	–
0.1	–	9 x 4	9.0 ± 0.0	0.01	
0.2	–	10 × 3, 11 x 2	10.4 ± 0.5	0.01	
0.4	–	11, 14, 15, 16	14.0 ± 2.1	0.01	–
0.2	5.0	20, 30, 31, 39, 41	32.2 ± 8.3	0.01	0.003
0.2	10.0	32X2,41,43	37.0 ± 5.8	0.01	0.001
0.2	20.0	33, 34, 54, 89, >150	72.0 ± 49.1	0.01	0.001

[a] ALS was injected i.p. one time 2 days before transplantation; IP-3082, i.v. by a 7-day osmotic pump.

Table III. *Combined effect of RAPA and ICAM-1 antisense IP-3082 on heart allograft survival*[a]

Treatment					
RAPA (mg/kg/day)	IP-3082 (mg/kg/day)	Heart Survival (days)	MST ± SD	*P*	*CI*
None	–	6,7X3,8,9,10	7.7 ± 1.4		
0.05		6 × 2, 7, 9 × 2	7.4 ± 1.4	NS	
0.1	–	10,11,20x2,21	13.0 ± 7.5	0.01	–
0.2	–	12, 14, 17, 18, 39	20.0 ± 10.9	0.01	–
0.1	5.0	23, 24, 33, 39, 43	37.4 ± 8.9	0.01	0.03
0.1	10.0	32 X 2, 36, 45	36.3 ± 6.1	0.01	0.02

[a] RAPA was delivered i.v. by a 7-day osmotic pump; IP-3082, as in Table II.

Table IV. *Combined effect of BQR and ICAM-1 antisense IP-3082 on heart allograft survival*[a]

Treatment					
BQR (mg/kg/day)	IP-3082 (mg/kg/day)	Heart Survival (days)	MST ± SD	*P*	*CI*
None	–	6,7x3,8,9,10	7.7 ± 1.4		–
0.5	–	9, 11 x2,14,15	12.0 ± 2.4	0.05	–
1.0	–	13, 16, 18, 19, 22	17.6 ± 3.4	0.01	–
2.0	–	15, 17, 20, 23, 25	20.0 ± 4.1	0.01	–
0.5	5.0	21, 24, 28, 29, 31, >100	38.8 ± 30.2	0.01	0.001

[a] BQR was delivered every second day by oral gavage; IP-3082 was delivered as in Table II.

administered for 7 days i.v. by osmotic pumps. Treatment of C3H recipients with 5 mg/kg/day IP-3082 for 7 days prolonged C57BL/10 heart allograft survival from 7.7 ± 1.4 days in untreated controls to 14.1 ± 2.7 days, producing an effective immunosuppression 5 to 7 days beyond termination of therapy. Similar results were obtained by using the 7-day anti-ICAM-1 mAb (27). Thus, the ICAM-1 antisense PS-oligo and anti-ICAM-1 mAb produced almost identical in vivo immunosuppressive effects.

Although these results are consistent with the antisense mechanism of immunosuppression, we cannot definitively conclude that IP-3082 acts by such a mechanism in vivo. The in vitro results suggest that IP-3082 acts in a sequence-specific fashion, that is, by blocking ICAM-1, but not VCAM-1 or G3PDH, expression. In the in vivo heart allograft model, the same IP-3082 prolonged heart allograft survival. A similar effect was not observed after treatment with a nonspecific IP-1082 or a scrambled IP-4189 control. Thus, both the in vitro and in vivo IP-3082 effects are sequence specific and, therefore, are consistent with antisense mechanisms. The IP-3082 therapy reduced leukocyte infiltration of heart allografts, as would be expected by the activity of an ICAM-I antisense PS-oligo. To inhibit ICAM-I expression, the IP-3082 may act directly on ICAM-I molecules present on the allograft or indirectly on the inflammatory cells that migrate to the graft. The latter effect may result in a decreased production of cytokines, leading to diminished ICAM-I expression and reduced infiltration of heart allografts. Experiments are in progress to elucidate the in vivo mechanism of immunosuppression by IP-3082 antisense PS-oligo in this complex transplant model. Furthermore, IP-3082 has shown activity in other inflammatory models, such as dextran sulfate-induced colitis and collagen-induced arthritis, thereby demonstrating that the same PS-oligo

Table V. *Combined effect of CsA and ICAM-1 antisense IP-3082 on heart allograft survival*[a]

Treatment					
CsA (mg/kg/day)	IP-3082 (mg/kg/day)	Heart Survival (days)	MST ± SD	P	CI
None	–	6, 7 × 3, 8, 9, 10	7.7 ± 1.4	–	–
2.5	–	6 × 2, 7, 8, 9	7.2 ± 1.3	NS	–
5.0	–	8, 9, 10, 11 × 2	9.8 ± 1.3	NS	–
10.0	–	8, 13, 14, 19, 21	15.0 ± 5.1	0.01	–
20.0	–	14 ×3, 17× 2	15.2 ± 1.6	0.01	
5.0	5.0	7, 8, 9 × 2, 10 × 2	8.8 ± 1.1	NS	14.1
10.0	5.0	6 × 3, 7, 14	7.8 ± 3.5	NS	51.0

[a] CsA was delivered by a 7-day osmotic pump; I-3082 was delivered as in Table II.

activity in vivo is not limited to a single model (C. F. Bennett and T. Geiger, unpublished observations).

The antisense ICAM-1 PS-oligo, IP-3082, interacted synergistically with other immunosuppressive agents, but not with CsA, to block heart allograft rejection. Most strikingly, the combination of IP-3082 and anti-LFA-1 mAb produced donor-specific transplantation tolerance. Similar results were described for the combination of anti-ICAM-I and anti-LFA-I mAb (27). Thus, both experimental procedures support the conclusion that ICAM-I and LFA- I are important for allograft rejection. ICAM-1 are expressed at low levels on normal endothelial cells, but not on myocytes or endocardium (42). During allograft rejection, ICAM-1 expression increases on endothelial cells and is induced on myocytes and the endocardium (43). In contrast, β_2 integrins are not expressed on cardiac tissue but are present on circulating leukocytes (42). There are at least two mechanisms by which ICAM- 1 and LFA-1 may contribute to the allograft rejection process, namely, by facilitating emigration of leukocytes into the cardiac tissue and providing the co-activation signals to the leukocytes (9, 11,14–18). In the absence of co-activation signals, T cells may become anergic or even clonally deleted (44, 45). In addition to ICAM-I and LFA-I, other adhesion molecules may facilitate emigration and provide co-stimulatory signals to leukocytes (9, 10, 42). In particular, three ligands (ICAM-1, ICAM-2, and ICAM-3) bind to LFA-I and three ligands (LFA-1, Mac-1, and CD43) may bind to ICAM-1. Therefore, inhibition of ICAM-1 or LFA-1 alone may not be sufficient to produce a strong in vivo effect. In contrast, the combination of two agents that inhibit ICAM- 1 and LFA- 1 expression produced a potent immunosuppressive effect, thereby inducing tolerance.

In addition, IP-3082 synergistically prolonged heart allograft survival when combined with ALS, RAPA, or BQR. These immunosuppressive modalities act in different fashions: ALS decreases the level of T cells, including alloantigen-specific T cells (34); RAPA inhibits the transduction of signals delivered by lymphokines (35); and BQR blocks the dehydroorotate dehydrogenase enzyme that is required for pyrimidine synthesis (37). Interestingly, all three agents acting by distinct mechanisms interacted in a synergistic fashion with IP-3082 to prolong allograft survival, as documented by CI values below 0.02. In contrast, CsA, which blocks calcineurin activity, thereby inhibiting synthesis of lymphokines by T cells (38), did not potentiate the immunosuppressive effect of IP-3082. It is possible that CsA may inhibit ICAM-1 expression and that this might diminish the activity of IP-3082. However, CsA is not very effective in mice; as much as 75 mg/kg/day was required to block skin allograft rejection (46), and 20 mg/kg/day CsA was needed to fully protect vascularized heart allografts from rejection (Table V). In vivo treatment with 15 to 45 mg/kg/day CsA did not affect alloantigen-induced performances of explanted T cells (39). Because mice are resistant to the immunosuppressive effect of CsA, it is unclear whether the antagonism between CsA and IP-3082 is a result of a pharmacologic or a pharmacokinetic reason. Similarly, control IP-1082 combined with CsA did not affect heart allograft survival. However, CsA is very effective in rats (47): 2 mg/kg/day CsA prevented heart allografts from rejection, 10 mg/kg/day CsA delivered for 14 days induced indefinite allograft survival. These in vivo results correlated with a dose-dependent inhibition of in vitro immune responses by T cells obtained from immunosuppressed recipients. Therefore, we plan to evaluate the interaction between CsA and rat ICAM-I antisense PS-oligo in rats.

ICAM-1 antisense IP-3082 seems to be well-tolerated at therapeutic doses without producing signs of toxicity. In fact, administration of IP-3082 to mice at a dose of 100 mg/kg/day q.o.d. for 14 days, did not produce any major toxic effects. Most important, in contrast with xenogeneic mAb, IP-3082 does not induce an antigenic response in animals (D. Kornbrust, unpublished observation). Furthermore, the pharmacokinetics of PS-oligos suggest that they are very efficient, and that they last long in vivo. Within 4 h, PS-oligos injected once i.v. accumulated predominantly in the liver (23%), kidney renal cortex (14%), medulla (3%), bone marrow (14%), and skin (13%). Metabolism of PS-oligos differed among organs. After 24 h, approximately 15% of the PS-oligo was intact in the liver, but as much as 40% was intact in the kidney (48). In blood, PS-oligos were found almost completely in the plasma fraction bound with low affinity to albumin and α_2-macroglobulin.

Experiments in monkeys were performed to examine the effectiveness of anti-ICAM-1 mAb (R6.5) to block rejection of kidney allografts (49). As in humans, ICAM-1 is expressed on the vascular endothelium in normal kidney. During rejection, ICAM-I expression increased on endothelial and tubular cells and leukocytes, and this increase correlated with massive infiltration of grafts. Anti-ICAM-1 mAb treatment decreased cellular infiltration and allowed administration of lower CsA doses. Recent clinical trials conducted in high-risk kidney allograft patients showed that adding mouse anti-ICAM-1 mAb (BIRRI) in a 14day postoperative period to the triple-drug therapy (CsA, azathioprine, and corticosteroids) improved 1-yr allograft survival from 56 to 78% (50). However, 16 of the 18 patients developed human anti-mouse Abs, which were detected between 3 to 14 days after completion of therapy with the BIRR1 mAb.

The PS-oligos used in this study for in vitro inhibition of ICAM-1 expression were added to the cultures in the presence of cationic liposomes. As previously shown, cationic liposomes enhance the activity of an ICAM-1 antisense PS-oligo (5 1-57). Interestingly, the same PS-oligo, IP-3082, that inhibited ICAM-1 expression in bEND.3 cells in the presence of cationic lipids, prolonged heart allograft survival when delivered to recipients by i.v. infusion suspended in saline without cationic liposomes. Similar observations have been noted with PS-oligos that targeted murine protein kinase C-α. An i.p. administration of this PS-oligo resulted in a selective reduction of protein kinase C-a mRNA in the livers of treated animals (58). Furthermore, several reports showed that local delivery of PS-oligos in the absence of cationic liposomes inhibited expression of the targeted gene (59-61). For example, a *c-myb* PS-oligo incorporated into a pluronic gel inhibited *c-myb* expression and smooth muscle cell proliferation in injured arteries (59). In another study, a *c-myb* antisense PS-oligo infused i.p. by a 7-day osmotic pump to *scid* mice bearing the human K562 myeloma leukemia tumor prolonged survival sixfold in comparison with untreated controls (62). Antisense, but not sense, PS-oligos complementary to the initiation codon of the nuclear factor-κB mRNA blocked development of fibrosarcoma in transgenic mice (63). To our knowledge, this study reports the first example of pharmacologic activity of an antisense PS-oligo by i.v. systemic administration in a model of a complex inflammatory process. In conclusion, our in vivo results demonstrate that antisense technology may proffer a new method of nontoxic and gene-targeted immunosuppressive treatment for organ transplantation.

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